

Getting rid of poor people's debts

The US administration may have bungled its well-meant plan to lift the burden of indebtedness from the shoulders of the developing world.

WILL Mr Nicholas Brady, the new US Secretary of the Treasury, be the next victim of the Bush administration's unpreparedness? Last Friday, Brady stood up in Washington to outline a solution to the problem of the debts owed by the poorest countries in the world to the richer countries and their commercial banks. Going further than his predecessor, Mr James Baker (Secretary of State in the new administration), Brady argued for a reduction of the debts the poor countries cannot afford to repay. The details are still vague, but Brady urged that the World Bank and the International Monetary Fund (IMF) should have extra resources so as to be able to buy out the interests of the commercial banks in some of their outstanding loans.

But who now can lend more? Only Japan. To its credit, the government of Japan announced its approval of Brady's suggestions even before his speech had been delivered. It seems to be implicitly agreed that, in the outcome, Japan will exercise much greater influence in the international lending institutions, which Japan appears to welcome. The US administration is more cautious, saying only that the details of the Brady plan are still to be worked out. But Brady's speech will by itself set in train events that neither he nor the White House can control.

That something should be done, and urgently, is plain. The total indebtedness of the developing countries is estimated at \$1.2 trillion, some 20 per cent more than the annual federal budget of the United States. More than a third of the debt is owed by Latin American countries, most of whom cannot make the required repayments; the rest is scattered through the developing world, which is hardly in better shape. The way in which the prospects for development are crushed by this burden of debt is plain. The Bush administration deserves credit for recognizing more plainly than its predecessor that the debt imbalance is now the most serious threat to international stability.

So why not forgive the debt? Commercial banks in the West have been compelled by their auditors to accept that the loans they have made are unlikely ever to be fully repaid; during the past two years, they have written off roughly a third of the debts they are owed. But that realism does not in itself relieve developing countries of their debts, which remain nominally repayable in full. The banks set out to collect what they can from whom

they can, pretending that the writing off of a third of the value of their loans is irrelevant in negotiations with individual borrowers. Without an agreement not yet reached between parties not yet identified, the banks have no choice.

But you cannot get water out of a stone, which is why the past year has been occupied with schemes for rescheduling developing country debt. The hope had been to stretch out debt and interest repayments over longer periods and, crucially, to lend the debtors extra cash to stimulate economic growth so that the attenuated debts would eventually be repaid in full. The first task has been relatively easy; the commercial banks lose nothing by rescheduling debts that would otherwise be repudiated. But extra cash has not materialized on a scale sufficient to allow the debtor countries to grow their way out of trouble. Instead, extra loans have often been barely enough to cover extra interest, and have been accompanied by economic constraints laid down by IMF which have often seemed intolerably onerous, whence the past month's disturbances in Caracas, the capital of Venezuela.

Brady's ambition is to short-circuit this process, which is admirable, and to do so within the context of a free market, which again makes sense. But how? This is where the plan tails off into thin air. In some form, funds provided by industrial countries (chiefly Japan) must be used to buy out the interests of the commercial banks in their doubtful loan agreements, presumably at a large discount. The banks would swap doubtful assets (their loans) for bonds whose face value is perhaps only a third as much, but which are somehow guaranteed by the international institutions. The obvious snag is that Brady's announcement gives debtor states an incentive immediately to diminish the book value of the loans they owe, while there can be no assurance that all the commercial banks owed money will take part in whatever scheme is eventually worked out.

It would have been better to put the horse before the cart, and to have negotiated the details of a workable scheme with those who will have to meet the costs (the commercial banks and the Japanese banking system) before giving other interested parties a chance prudently to escape their obligations. It is also plain that there can be no permanent settlement without an understanding of the extent to which the poor countries can expect to work off their debts by trading freely with their creditors. The



United States has a particular, but urgent, need to devise a coherent policy on relations with Latin America, shamefully neglected in the past eight years. But, now that Brady has spoken, all this has to be arranged by tomorrow or, at the latest, the day after tomorrow. This is a slipshod way in which to deal with such an important matter. □

All greens now

Politicians anxious about the environment seem no more ready than their predecessors to make prudent choices.

THE most obvious intellectual weakness of the environmental movement, already apparent in its first flowering in the late 1960s, is its habitual failure to tell what is important. So much is now clear in the general response to the otherwise welcome recognition by senior politicians that the greenhouse effect must be taken seriously. Suddenly, it seems, a whole range of public policy is legitimately to be dominated by what are called ecological imperatives. One result will be that public and private resources will be squandered needlessly. Another is that our capacity to deal with the important problems will be seriously impaired.

Here is a sample of how the wind is blowing. A few months ago, the government of the Netherlands decided to require that new motor cars should meet emission standards more stringent than those agreed by the European Community in a famous compromise reached only with the greatest difficulty four years ago — and to which the Dutch were then a party. The result (unless the decision is overturned by the European Court of Justice) may be that Dutch motor-cars will in future emit less pollution while the rest of Europe reverts to its older ways. In West Germany (see page 194), self-evidently innocuous experiments with genetically engineered plants must be carried out in a limbo that will be removed only when there is a legislation on the subject, likely to be the most restrictive in Europe. In Britain last week, a plan to build a railway link between the channel tunnel and the rest of Britain was hastily rerouted to meet environmental objections, at an estimated extra cost of £500 million. Mrs Margaret Thatcher, the British prime minister, is both consistent and right to say that the extra cost must be met by those who use the railway, which is unlikely to be commissioned until several years after the tunnel. But, on the Principle of the Conservation of Money, the extra £500 million spent to preserve the gentility of rural Kent will be £500 million less to spend on other worthwhile causes.

Collective sanity requires a better sense of proportion. All environmental protection in which governments are involved is at bottom a public purchase — one made on behalf of a whole community with resources drawn from it either by taxation or by the payment of user fees. The government of the Netherlands, for example, has implicitly arranged that the costs of reducing exhaust emissions

will be met by Dutch motorists, who will pay more for their cars and more for their fuel. (The Netherlands may hope to recoup some of the extra cost if Dutch motor-manufacturers find they have an edge in the domestic market-place over their competitors.)

The British railway to the channel tunnel is in the same case. The preservation of a corridor through Kent has been judged worth £500 million, which the operators of the railway will have to find and then recover by means of a tax (extra fare) on the railway's users. Meeting the extra capital cost out of public funds (which the British government will be asked to do) would simply redistribute the burden.

Sadly, in all of these decisions, the communities on whose behalf governments have acted (or, in the case of West Germany, failed to act) have not been presented with a choice. Yet the extra cost of the British railway far exceeds what would be the cost of drawing up and enforcing legislation to make British farm foodstuffs free from *Salmonella* infection but may be a more prudent investment. So should not the governments committing these substantial sums of money ostensibly in the public interest ask the communities they are supposed to serve what choice they should make between the alternatives? Surprisingly, even the British government, which likes to think of itself as strong, seems to prefer to be pushed about in one direction or another as local or otherwise special interests make themselves heard.

The conversion of people such as Thatcher to a proper concern about the greenhouse effect should have engendered a more resolute view of environmental costs, but has not. Yet the crude arithmetic of the case is as accessible as the back of the nearest envelope. Suppose there is eventually a carbon dioxide convention, and that countries such as Britain have to generate all their electricity from fuels other than hydrocarbons. That could entail building 50 million kW of nuclear generating capacity over say 20 years, which would work out at an investment of £5,000 million a year if the extra cost of nuclear generating capacity is merely £2,000 a kilowatt. That simple arithmetic does not imply that nuclear capacity is the only alternative, but unconventional sources of electricity would probably on present form be more expensive, nor does it allow for the replacement of fossil fuels in other energy-consuming machinery, vehicles for example.

In short, if it should eventually be necessary to reduce carbon dioxide emissions, the costs are likely to be orders of magnitude greater even than the sums now being frittered away on the railway to the channel tunnel. One way of responding to that dilemma is to say that if measures to combat the greenhouse effect are likely to cost several thousand million pounds a year, £500 million or so for Kent is mere chicken-feed. But that is also the route to national impoverishment. The more prudent course is to require than people should choose. Thatcher, above all politicians of her persuasion, should be eager to remind people that that is not merely their right but their duty. □

Changes in misconduct investigation planned

- Present procedures declared inadequate
- New legislation may obstruct plans

Washington

THE US Public Health Service (PHS) has decided to create a new mechanism for dealing with scientific misconduct by establishing an Office of Scientific Integrity in the office of the director of the National Institutes of Health (NIH).

This move comes after months of internal debate on how best to tackle the growing concern that current procedures for investigating alleged scientific misconduct are inadequate. But the rapid implementation of the new plans may not be easy, for they seem to be at odds with legislation about to be introduced in Congress.

Virtually nobody is satisfied with present procedures for dealing with scientific misconduct. Investigations have taken much longer than expected, there have

Although the new office of scientific integrity will be located in the office of the NIH director, it will be responsible for investigations and procedures for all PHS agencies, including the Alcohol, Drug Abuse and Mental Health Administration and the Centers for Disease Control.

Congress has put pressure on PHS to make changes in the way it deals with misconduct. Last year, there were several hearings that drew attention to problems

in current procedures. Earlier this year, Representative Henry Waxman (Democrat, California) asked the Inspector General of the Department of Health and Human Services to determine whether PHS was putting sufficient resources into its investigations.

Legislation is at present being drafted by Waxman and Representative John Dingell (Democrat, Michigan) — head of the powerful House of Representatives Energy and Commerce Committee — that would also create an office of scientific integrity, but place it in the assistant secretary's office, not at NIH. It seems certain that Congress will require assurance of a serious commitment to investigating misconduct before it agrees to let NIH retain primary responsibility for that role.

Joseph Palca

US SCIENCE POLICY

Who should decide policy?

Washington

THERE is a need for a new government body to set national goals in science and technology in the United States. That much was agreed at hearings held over the past two weeks by the House of Representatives Subcommittee on Science, Research and Technology and the Senate Committee on the Budget. But the form that the new policy-making body should take was a matter of great debate.

The consensus that the United States lacks a coherent national policy in science and technology has arisen for two reasons. In recent years, emerging big projects, such as the sequencing of the human genome and the study of global climate change, have cut across government agency boundaries. But there is no central government body to determine the relative importance of these projects, and the budgets they should command. On another front, Congress and industry are realizing that the United States is being left behind in development of key new technology, such as high-definition television (HDTV); that is also perceived as due to a lack of a guiding national policy.

Former presidential science adviser George Keyworth revived the idea of a new department at the House hearing. But several subcommittee members and witnesses dismissed this idea. William Carey, former executive director of the American Association for the Advancement of Science and a former assistant director of the Bureau of the Budget, says a new department would be "crippled" by lack of support from other departments and agencies. And when Clyde Prestowitz of the Carnegie Endowment made a similar suggestion at the Senate hearing, Senator Ernest Hollings (Democrat, South Carolina) said "We would all be dead and gone" before such a department

is realized.

Carey instead favours proposals recently put forward by the National Academy of Sciences which call for collaboration between the Office of Management and Budget and the president's science adviser in determining priorities, objectives and budgets of large projects. A step in that direction has already been taken by the Committee on Earth Sciences of the Federal Coordinating Council for Science, Engineering and Technology, which has for the first time prepared a cross-agency budget for research on global climate change in 1989 and 1990.

Under the academy proposal, cross-cutting research activities would be reviewed by budget and appropriations committees in Congress before being broken down for consideration by individual agencies. Another approach, put forward by Lawson Crowe (University of Colorado), is to establish a national commission, composed largely of scientists, that would rank large-scale projects according to scientific merit, social benefit and "programmatic implications".

But most subcommittee members seemed more concerned with mechanisms for strengthening economic competitiveness than with scientific policy. Almost every witness was asked what should be done about HDTV, which is fast becoming the touchstone in Congress of the competitiveness of US industry.

The hearings are almost certainly a harbinger of a year of debate in Congress over the question of how to set science and technology policy. The same issues are certain to come up again when President George Bush's plans for giving his science adviser the status of an assistant to the president and for appointing a panel of independent advisers are put forward as legislation. **David Swinbanks**

Memo to John Tower: Perhaps this one's not quite right for you either...



been squabbles over who should investigate cases and Congress has felt that NIH may lack the will to tackle the difficult issues that inevitably arise. A report from the Institute of Medicine urges that encouraging proper research conduct is an important part of preventing misconduct (see *Nature* 337, 588; 16 February 1989). Accordingly, the new office at NIH will be responsible not only for investigating alleged misconduct but also for promoting high standards of laboratory practice.

The new office will make recommendations to the Assistant Secretary for Health, who is the head of PHS. The plan also calls for an Office of Scientific Integrity Review in the office of the assistant secretary to oversee investigations by the NIH office and to help develop policies for dealing with issues of integrity and misconduct.

Industry struggles on despite opposition

Washington

Will nuclear power play a role in the future energy plans of the United States? The Nuclear Power Oversight Committee, a nuclear power industry body, issued a white paper (policy document) last week calling for an easing of government regulations so to encourage the building of new nuclear plants. But industry analysts say nuclear power is dying in the United States, not because of regulation but because the industry has lost economic and political viability.

James O'Connor, chairman of the committee, says that growth in demand for electricity in recent years has far exceeded even the power industry's projections and that the United States is now running "perilously close to capacity margins that leave very little room for error". He points to the emergency measures, such as voltage reduction, that many utility companies had to take during last summer's heat wave as a sign of the serious supply difficulties that lie ahead.

If demand for electricity in the United States continues to grow at 2–3 per cent a year, 120–220 large new nuclear power plants will have to be built by the turn of the century. The white paper argues that new plants could offer the most cost-effective new source if the government were to ease regulatory controls by, for example, introducing a single-licence system instead of, as now, requiring utility companies to obtain both a construction and an operating licence for new plants.

But Christopher Flavin, vice-president for research at the Worldwatch Institute, who has been monitoring the nuclear industry for the past decade, says the industry's figures for the cost of nuclear power are based on existing plants both old and new. Since the Three Mile Island accident, increased safety requirements have quadrupled plant construction costs and nuclear power is now more than "twice as expensive as competing sources". But O'Connor argues that the biggest additional cost in building new plants is delay due to complex regulatory controls.

Concern about global climate change has prompted Congress to reassess the pros and cons of nuclear power, an energy source free from carbon dioxide emissions, as the industry is ready to point out. But Flavin says that, if the new legislation introduced by Congress to deal with global warming is accepted, there will be a "stampede" towards the development of renewable energy sources, not nuclear power.

Public opposition to nuclear power in the United States remains widespread. Legislators introducing global warming bills have attenuated or omitted references to nuclear power to gain wider acceptance.

David Swinbanks

Legacy of Three Mile Island

Boston

A DECADE after the partial meltdown of the Three Mile Island (TMI) Unit 2 reactor near Harrisburg, Pennsylvania, on 28 March 1979, cleaning up continues at a cost of \$1,000 million — nearly \$300 million more than the cost of building the reactor in the first place. But the implications of the accident for the commercial nuclear industry are almost certainly far greater.

So far, workers have removed most of the damaged nuclear fuel from the Unit 2 reactor vessel. This has been accomplished by a team of more than 1,000 people, including a group operating specially designed equipment from a rotating platform above the reactor vessel. Working almost continuously since October 1985, the clean-up team has removed radioactive debris ranging in size from gravel-like material to a fused hard crust, up to 5 feet thick, formed from the molten fuel rods during the accident.

If all goes according to plan, the 'defuelling' process should be complete by the end of 1990, when Unit 2 will be shut and monitored for approximately 30 years, until the end of the planned lifespan of the operating Unit 1 reactor nearby. A full decommissioning and dismantling of Unit 2 will be postponed until the job can be undertaken for both reactor units together, according to Doug Bedell, spokesman for GPU Nuclear, TMI's owner.

Bedell emphasizes that enough material has been removed to dispel any chance of "a critical event", but he acknowledges that, because residual caesium has permeated the basement walls, "unacceptable" and potentially dangerous levels of radiation will still be left in the basement area of the Unit 2 reactor until decommissioning begins next century.

Since the clean-up began, 150 tons of highly radioactive debris has been hauled away from the reactor. But because there is not yet a functioning repository for high-level commercial nuclear waste anywhere in the United States, the debris from TMI continues to be stored "temporarily" at the National Engineering Laboratory in Idaho where researchers have studied the material to reconstruct the precise course of the accident. A plan to evaporate the remaining 2 million gallons of radioactive water at the TMI site still awaits approval from the Nuclear Regulatory Commission.

The accident at TMI Unit 2 began after only 11 months of operation when a pump providing feedwater to the reactor core clogged during full power operation. After the plant shut down automatically, a relief valve opened to diminish the built-up pressure and temperature in the

reactor, letting out steam and water.

Undetected by the operators, this valve remained open for 2 hours, allowing the escape of nearly a million gallons of the coolant water covering the reactor fuel. As approximately half of the reactor core became uncovered, the radioactive water overflowed designed catchments, collecting in the basements of the reactor and auxiliary buildings. Without the coolant water, temperatures in the pressurized-water reactor rose quickly, causing some of the uranium fuel to melt.

The accident at TMI prompted a widespread response in the nuclear industry. Within weeks of the disaster, the Electric Power Research Institute (supported by the utilities) established an organization called the Nuclear Safety Analysis Center (NSAC) to analyse the accident and to inform other utilities of any future malfunctions or other information that could be useful.

Later that year, the industry also formed the safety-oriented group called the Institute of Nuclear Power Operations (INPO). Among its other functions, INPO runs a computer network linking nuclear power plant operators with experts at INPO and NSAC to provide instantaneous advice and information. According to William Layman, since the accident at TMI, the industry has learned that "the price of safety is eternal vigilance".

But although the industry can point to strengthened safety procedure in the aftermath of the TMI accident, most agree that the incident's greatest legacy is a sustained level of lingering public fear and opposition to nuclear power in the United States.

More than 2,000 lawsuits claiming health and psychological damage from the TMI accident are still pending, despite measurements after the accident that showed that the highest possible whole-body dose to any one individual was less than 100 millirem, or only slightly greater than the average most people are exposed to from medical and dental radiation in a year.

Most observers agree that the TMI accident was a clear turning-point for the US nuclear industry, although poor performance, steeply rising construction costs and slowly growing demand for electricity may earlier have presaged the industry's decline. Although performance has improved and dependence on nuclear power as a fraction of energy supply in the United States has since grown, no plants have been ordered since the accident and all of the 47 units ordered in the United States since 1974 have been cancelled.

Seth Shulman

Plan to close schools resisted

London

THE review of veterinary science at British universities, published earlier this year, has been so fiercely attacked that it seems unlikely that its proposals will be implemented unchanged. The review (see *Nature* 337, 294; 26 January 1989) recommended that the veterinary schools at Glasgow and Cambridge should be closed.

Representatives of the veterinary profession now say that the closures will increase manpower shortages, lead to a lowering of standards in the veterinary profession and persuade more British researchers to go to the United States to work. In the universities, even those who agree that some reduction of capacity would be wise say the working party has picked the wrong targets.

The review is one of a series being carried out for the University Grants Committee in an attempt to decide how to use most efficiently the limited financial resources of Britain's universities. The working party for the veterinary sciences, whose chairman is Sir Ralph Riley, was told that a maximum intake of 335 students a year will be necessary to meet future manpower requirements and was asked to frame its recommendations accordingly.

To ensure that clinical sciences are taught with satisfactory spread and depth, the working party concluded that each school should have at least 36 clinical teachers. With some redistribution of posts, four schools, instead of the present six, would be sufficient. In order that the remaining schools are as widely spaced geographically as possible, the working party concluded that one of the two

schools in Scotland and one in the South-East of England would have to be closed down.

Because there is room for expansion at Edinburgh, the Glasgow school was selected for closure. But the review says that the Edinburgh school should cease to exist in name, and that a new Scottish school would be formed by a merger of the two.

The Royal College of Veterinary Surgeons and the British Veterinary Association last week launched a furious attack on these proposals. They argue that future manpower needs require an intake of 400–500 students a year, which four schools could not provide. The registrar of the college, Alistair Porter, says the shortage of veterinary surgeons has already brought an influx of manpower from other countries.

The president of the Royal College, John Parsons, says that researchers would prefer to move to the United States rather than agree to be relocated in Britain. Since the announcement of the proposed closures in January, job offers have started coming in to researchers at Glasgow and Cambridge.

But those at the highly regarded department of veterinary pathology at the University of Glasgow are unlikely to be lured away. Even if the Glasgow school does move, there are provisions in the Riley report for this department to continue to exist at the University of Glasgow. The campaign against closure of the veterinary school in Glasgow has won widespread support in the local community: a petition with more than 300,000 signatures is to be sent to the prime minister.

Christine McGourty

Students hang back, researchers may quit

Sydney

THE poor job market for Australian scientists is apparently starting to cause problems for the future of Australian science. The signs of trouble are clear from the figures for university enrolment for this academic year. According to a report by Australian Vice-Chancellors Committee, universities have had to lower entry standards to find enough science students. At the University of Sydney, entry scores in the science faculty dropped from 320 out of a possible 500 in 1987 to 300 this year, the lowest entry aggregate for any faculty. The University of Melbourne was forced to drop entry requirements from 295 in 1988 to 279 this year in order to fill its quota of science students.

But even those already on the career ladder are finding the climb tough. Seventy-five per cent of non-tenured Commonwealth Scientific and Industrial Research Organisation (CSIRO) scientists are unhappy with their career prospects, according to a survey by the recently formed Australian Science Action group, headed by Dr Greg Tanner, a CSIRO plant scientist. Forty per cent of the total sample size of 175 say they are prepared to leave Australia. Of these, 28 per cent indicated that they would pursue science overseas, while the rest say they would leave science altogether.

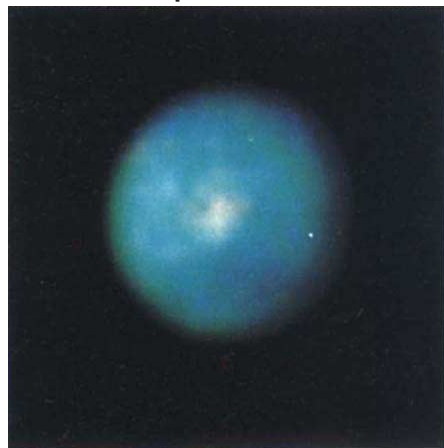
The CSIRO study shows that disillusion is greatest among younger postdoctoral researchers without tenure. The disaffection is particularly striking among biologists and chemists; those in the physical sciences less commonly regarded their prospects as poor. Women scientists with families are also discouraged about staying in science because of the reduced number of part-time positions available.

Tania Ewing

NEPTUNE FLY-BY

Voyager's first glimpse of distant planet raises NASA's hopes

PROSPECTS for Voyager 2's fly-by of Neptune on 24 August are encouraging, according to the mission controllers at the Jet Propulsion Laboratory in Pasadena. The colour images shown here, taken on 23 January when Voyager was about 185 million miles (309 million km) from the planet, already show detail down to 3,500 miles (6,000 km), better than that available from the best telescope images from Earth. The fact that distinct cloud features are visible when the spacecraft is still so distant suggests that images obtained during August will show far more detail than was visible in the atmosphere of Uranus, which Voyager 2 encountered in January 1986. The natural colour of Neptune, like that of Uranus, is a pale blue-green, caused by methane gas in the atmosphere, which absorbs red light. The images here were obtained through violet, clear and orange filters. □



Two images of Neptune taken about 2 hours apart. The bright cloud feature visible near the centre of Neptune's disk in the earlier (left-hand) image has moved to the right edge of the planet in the later image. This is in line with the 17- to 18-hour rotation calculated from Earth-based observations. A dark band of clouds, similar to structures seen on Jupiter, Saturn and Uranus, can be seen encircling the southern pole. □

Clinical researchers at odds

Washington

A FEDERAL appeals court has written the latest chapter in a bitter dispute over plagiarism between a former faculty member of Albert Einstein College of Medicine in New York and her mentor, now vice-chairman of nuclear medicine at the medical school. A three-judge panel overturned a lower court's ruling, and declared that Leonard Freeman violated copyright law when he put his name on a document prepared by Heidi Weissmann, but Freeman's attorneys have appealed against the latest decision. The case threatens to drag on.

Told one way, there seems little doubt that Freeman misappropriated Weissmann's work. In August 1987, Freeman was invited to present work on liver imaging in a review course at Mount Sinai School of Medicine in New York. Freeman took a syllabus prepared by Weissmann for a similar course sponsored by the Radiological Society of North America in 1985, deleted Weissmann's name from the manuscript and put his in its place, and changed the title from "Hepatobiliary Imaging" to "Gastrointestinal Nuclear Medicine Hepatobiliary Imaging". The same syllabus appeared in a book published in Taiwan under Freeman's name after a symposium in December 1986 in the Republic of China.

But the case can also be read differently. Weissmann and Freeman began their association when she was a resident at Einstein in 1977, and had collaborated on liver-imaging work since 1980. After finishing her residence, Weissmann became a faculty member at Einstein, working in Freeman's division.

The 1985 syllabus used by Weissmann was largely drawn from previous versions of the syllabus which were jointly produced, but which differed in some references, illustrations and some new text. Furthermore, when Weissmann filed her suit to prevent Freeman from presenting the syllabus at Mount Sinai, she was in the midst of a lawsuit accusing Einstein of sexual discrimination over issues of salary and sabbatical leave.

A district court judge in New York agreed with Freeman's contention that the syllabus was a product of collaborative research, and a continually evolving 'stock' piece to be used when either was called upon to teach a course in hepatobiliary imaging techniques. The judge ruled that Freeman should be considered a joint author of the syllabus, and therefore could not be guilty of violating copyright of it. He also ruled that Weissmann's modifications to the 1985 syllabus were insufficient to support separate copyright protection and that, even if Freeman was not considered a joint author of the syllabus,

his intended use of the document fell under the 'fair use' doctrine as it was intended for teaching and not personal profit.

But the appeals court saw things differently. In a 2-1 decision, the court ruled that Freeman's use of the syllabus was a violation of copyright. The majority opinion states that the decision of the lower court "stands copyright law on its head" by concluding that joint authorship of prior work "automatically makes the two joint authors co-owners of the derivative work". The court goes on to state that originality is rightly prized in law, and alludes to Dostoevsky's example in *The Idiot* that a person's worth in revolutionary Russia "did not depend so much on money or position as it did on one's originality". The appeals court also ruled that, although he did not stand to gain financially from the copyright infringement, Freeman did gain in stature professionally by passing Weissmann's work off as his own.

Einstein and Montefiore Medical Center, where both worked, have been firmly behind Freeman throughout the dispute. Part of Freeman's legal costs have been met by his employer. An open letter from Montefiore's president Spencer Foreman following the lower court's ruling last May declared that the "Federal District Court has completely vindicated our colleague Leonard M. Freeman", and refers to Weissmann as "a former Montefiore employee and former student", rather than as a former faculty member. The letter ends with "I know you are all as pleased as I am with the decision reached by the federal court".

Weissmann is now out of work. The medical school maintains that she resigned when she failed to return to work after a sabbatical which ended in September 1987. Weissmann says she was fired because when she tried to go to her office in August of that year, days after taking legal steps to prevent Freeman from handing out the syllabus at Mount Sinai, hospital security guards confronted her and demanded that she hand over her office keys. Freeman, meanwhile, has been promoted to vice-chairman of his department.

Freeman says the dispute should never have gone to court. Weissmann says she by-passed university mechanisms for resolving disputes in the copyright case because she did not think the university would give her case adequate attention.

Einstein has had a poor track record in its attitude towards female faculty. It has been the object of a class action suit filed in the 1970s alleging sexual discrimination on salaries that has still not been settled. Eugene Girden, a lawyer for Freeman,

Sterilization protests

Munich

A PROPOSED new law that would make it legal to sterilize people judged mentally incompetent without their consent has prompted a vigorous protest in West Germany from those who fear the law might some day be used to justify enforced sterilizations of a broader class of people.

The provisions on sterilization are part of an otherwise welcome reform of the rights of mentally disabled people. The legislation would ban the sterilization of minors, and has been well received on that account across the political spectrum, especially because of its emphasis on counselling and support for people unable to speak for themselves. But critics say that the legislation also contains a potentially dangerous loophole that might at some future stage be widened by allowing the sterilization of adults who are mentally incompetent.

Hitherto, up to 1,000 sterilizations a year have been carried out in West Germany on people — minors and adults — deemed legally incapable of handling their own affairs. Many of these sterilizations are done at the request of parents of mentally handicapped children. Under West German law, incapacitated people have had no legal recourse against the procedure.

The new legislation permits the sterilization of people who do not specifically object in cases where pregnancy would create an "emergency situation". Any objection on the part of the person in question would result in the cancelling of the sterilization.

The Green Party, a judges' organization and handicapped rights groups have protested against the law and are preparing to fight it in parliament. The current law, which dates back to 1900, deprives mentally incompetent adults of all rights, leaving them the equivalent of a child in legal terms. The new law was meant to be a landmark in a series of social programmes that have been staggering through parliament in recent months. The proposal has been sent to both houses of the West German parliament for discussion. The government hopes to gain approval for the law by the end of this year. Steven Dickman

says it is ironical that Weissmann is suing Freeman, as he was one of her strongest supporters at the university.

Weissmann's case has been taken up by the National Coalition for Universities in the Public Interest, a Washington watchdog group, which intends to file new charges in Weissmann's discrimination suit still pending in New York State court. Leonard Minsky, the coalition's executive director, argues that Weissmann's experience is just one of a series of cases involving plagiarism, sexual discrimination and conflict of interest in US universities.

Joseph Palca

Invitation from Soviet centre

London

PLANS to found an international research centre on Lake Baikal in south-east Siberia have been formally agreed in the Soviet Union, where the Soviet Academy of Sciences has deputed responsibility for the project to its Siberian Division. The division is now inviting putative members of the centre to declare their interest, and plans to hold a constitutional conference at Irkutsk in the second half of this year.

The formal announcement, contained in a cable to the Editor of *Nature*, is as follows: The Praesidium of the Siberian Division of the Academy of Sciences of the USSR announces its intention of organizing the Baikal Centre for Fundamental Ecological Research — an organization open for the collaboration of Soviet scientists and scientists of all other countries in studies of the greatest ancient freshwater lake, Lake Baikal.

The facilities of this centre are sited, and will be further developed, at Listvyanka, on the shore of Baikal, and in Irkutsk, 70 km away. The Limnological Institute at Irkutsk will provide research vessels (six ships), laboratory rooms, expeditions, equipment, hotel rooms, etc., as agreed with participating groups and contracted for.

It is proposed that the scientific prog-

ramme of the centre should include the following topics:

- Interdisciplinary studies of the Baikal ecosystem using the techniques of hydrodynamics, hydrochemistry, hydrobiology, remote sensing and mathematics in order to create a conceptual working model of this ecosystem.

- Studies of the speciation of the unique Baikal endemic complex of some 1,500 organisms and of the evolution of their nucleic acids and proteins.

- Investigation of the global transfer of elements and priority pollutants.

- The development of methods of environmental monitoring.

- Geological studies of Lake Baikal and of the history of its formation.

The Baikal International Centre will be operated by an international board of directors appointed by the founding parties. These will include the Siberian Division of the Academy of Sciences of the USSR, other Soviet organizations and organizations from other countries which agree to support the centre financially and to participate in its activities.

The board of directors will consider proposals for research projects and will select those to be carried out in each succeeding year on the basis of the reports of international referees. It will also decide what proportion of the budget will be devoted to each project selected in advance of each year's research.

Potential founding members of the Baikal International Centre — scientific institutes, scientific societies and international agencies — are hereby invited to open discussions with the Limnological Institute to clarify the framework of the agreement with the Siberian Division regarding participation in and support for the activity of the centre. Please write or cable to Professor M. A. Grachev, Lermontova 281, 664033 Irkutsk, USSR.

Inquirers will be supplied at their request with a draft charter of the centre, a list of the scientists who have already endorsed the concept of the centre and who have participated in collaborative research at Baikal as well as with any other relevant information.

It is hoped to hold a conference with representatives of the founding members of the centre at Irkutsk in the second half of 1989.

The cable is signed "Praesidium of the Siberian Division of the Academy of Sciences" and has been verified by telephone in a conversation with Professor Grachev. A general account of the Siberian Division's plans will be found in *Nature* 337, 111; 12 January 1989. □

■ See page 209 of this issue for Professor Grachev's account of the canine distemper virus infection of Lake Baikal seals.

Third shuttle launch

Washington

THE third space shuttle launch since flights resumed took place last Monday (13 March) when the shuttle *Discovery* lifted off from Cape Canaveral in Florida. The lift-off was delayed one hour and forty minutes by poor weather conditions at the launch site. The mission's primary goal is to deploy a third tracking and data relay satellite that will complete a system of communications satellites in geosynchronous orbit that facilitate much higher data rates from spacecraft to ground stations. The new system is essential to make optimal use of data from the Hubble Space Telescope to be launched in December. This telescope was costly and expectations of it are high (see page 199).

The next shuttle mission, scheduled for 28 April on the shuttle *Atlantis*, will launch the *Magellan* spacecraft, a planetary probe bound for Venus. J.P.

Blaser to retire from PSI

Munich

J. P. BLASER will be retiring in the spring of 1990 as director of the Swiss Paul Scherrer Institute (PSI) for nuclear research in Villigen, near Zurich. The institute is currently seeking a replacement. Blaser, 66, was formerly director at the Swiss Institute for Nuclear Research, which was fused with the Federal Institute for Reactor Research at the end of 1987 to form PSI. S.D.

New authority in view

London

The task of reaching international consensus on how to combat global warming was tackled last week at an international conference on the environment held in the Hague, and organized by the governments of France, the Netherlands and Norway.

The declaration at the end of the conference calls for a new authority to be set up in the United Nations to be responsible for combating global warming. It says that the authority should undertake or commission scientific research and ensure the distribution of information between nations, including the sharing of technology. The authority would set standards for protection of the atmosphere and monitor compliance. But the conference decided not to endorse the imposition of economic sanctions on offending nations; measures to promote compliance would be referred to the International Court of Justice.

It was agreed, however, that developing countries should be compensated for any burden placed on them by the regulations, according to their responsibility for pollution. The organizers were criticized for their selective invitations — the United States and the Soviet Union were among those not invited. But the Dutch say all nations were invited to sign the declaration. C.McG.

US UNIVERSITIES

MIT studies relations with Lincoln Laboratory

Boston

THE Massachusetts Institute of Technology (MIT) has announced the formation of a committee to investigate the university's relationship with the federally sponsored Lincoln Laboratory in Lexington, Massachusetts. The laboratory's \$386-million annual budget is paid by the United States government, but the institute operates the facility.

At the core of the inquiry is the laboratory's emphasis on defence-related research involving radar and advanced electronics. Much of the work at the laboratory is classified, despite a university policy that eschews classified research on campus.

Paul Gray, president of MIT, says the new committee, headed by Joel Moses, chairman of MIT's electrical engineering and computer science department, will "explore alternative methods" for administering the Lincoln Laboratory. Gray has asked the committee to report by September. The group's twelve members also include Jerome B. Weisner, former president of MIT, Francis E. Low, former MIT provost, and Walker Morrow, Jr, director of Lincoln Laboratory. Seth Shulman

SPOT on, but Landsats off?

Paris & Washington

US and French remote-sensing satellites are facing very different futures. EOSAT, the Earth Observation Satellite Company that operates the two orbiting Landsat satellites for the National Oceanic and Atmospheric Administration (NOAA), has been instructed to begin shutdown procedures for both satellites, putting them in a stand-by mode from the end of this month. By contrast, SPOT, the French Earth-observing satellite, had its third anniversary in space marked by a book about its history*.

The chief reason for cutting down the Landsat programme is money. Both Landsats 4 and 5 have exceeded their design lives, and although they are still providing useful information, neither the White House nor Congress has been interested in spending money on upgrading them.

NOAA is obliged to provide EOSAT with money to operate the two satellites, but with its own budget thinly stretched, the agency has been looking to EOSAT revenues to help defray expenses. EOSAT has responded by arguing that current Landsat revenues are needed to prepare for Landsat 6, now due to be launched in 1991.

EOSAT maintains that \$9.4 million would allow it to keep operating the two Landsats until the end of the fiscal year (at the end of September). Although the details of a package have not been worked out, NOAA assistant administrator Thomas Pyke last week told a congressional subcommittee that the Bush administration is committed to keeping Landsats 4 and 5 operating until a policy review of the programme is completed. The new National Space Council, chaired by Vice-President Dan Quayle, is coordinating the effort to find money in the budgets of federal agencies that use Landsat data.

Although still operational, SPOT is beginning to show signs of ageing, and a decision will soon have to be made whether to launch its younger sister, SPOT 2, as planned, later this year. To delay the launch would save money in the short term for the financially stretched French space programme, but the gamble could be too risky.

Seen as a white elephant by all other European countries except Sweden and Belgium, SPOT was financed and built almost entirely with French money. Now, with more than 300,000 high-resolution images in its owners' archives for sale to anyone who wants them for as little as \$450, SPOT has convinced even sceptics of its value.

The US Department of Defense came

to respect SPOT two years ago, when *Aviation Week and Space Technology* published photographs bought over the counter from SPOT Image Corporation that showed Soviet naval installations. The Pentagon was worried by the realization that anyone could also buy pictures of defence installations in the United States.

SPOT 2 should have been launched last year, but a long setback in the European Ariane rocket launch programme meant that SPOT 1 was kept in service. Although it still sends 700 images each day, one of its two data buffers is out of action and a faulty sensor in each of the two high-resolution cameras has reduced image quality. If the other buffer should fail,

ENVIRONMENTAL RELEASE

Pink petunias sow controversy in West Germany

Munich

A CONTROVERSIAL proposal to plant 40,000 genetically engineered pink petunias has been approved by a West German panel, but the future of the experiment remains in doubt. It is the first time that release of a plant containing recombinant DNA has been approved in West Germany. But the federal Health Ministry must pass judge-



Saedler — expressing a lack of optimism.

ment on the experiment before planting can begin.

The experiment itself is not expected to pose a danger to the environment, but opponents fear that it will convince the public that all release experiments are harmless. The experiment is important because it should force better definition for each phase of the now vague licensing procedure. Challenges to approval could come from one of several offices within the

details of some of the most strategic parts of the globe would be unavailable, and the satellite's commercial value would be drastically reduced.

The French space research centre (CNES) has booked a place for SPOT 2 aboard Ariane flight 34, due in September. But the French space programme is heaving under the burden of recent financial commitments to the European Space Agency and CNES would like to delay the launch so long as SPOT 1 is working well. Although CNES says that SPOT 2 could always be launched at short notice, if the flight 34 option is not taken up, a failure in SPOT 1 would leave France with only an exotic photograph album to sell until another launcher is free, which might not be for several months.

Peter Coles & Joseph Palca

federal Health Ministry, the federal Environment Ministry or the Land of North Rhine-Westphalia.

Biological release is a controversial issue in West Germany, as shown by the storm of protest that greeted Bayreuth geneticist Walter Klingmüller when, in 1987, he carried out an unauthorized experiment with nitrogen-fixing *Rhizobium* bacteria (see *Nature* 328, 568; 1987). In 1985, there was a bomb attack on the Max Planck Institute (MPI) for Breeding Research in Cologne, which has planned the petunia experiment.

Release experiments must now be undertaken in the legal vacuum preceding the long-awaited introduction into parliament of a 'basic law' for regulating genetic engineering. The Central Commission for Biological Safety, the body that unanimously approved the petunia experiment, can request only voluntary compliance with its regulations.

The experiment will demonstrate the behaviour in petunia of so-called 'jumping genes' (transposons). A maize gene has been introduced into the petunia that turns them an unnatural pink colour. If a transposon should jump into the middle of this coloration gene, it is expected to leave parts of the flower white. Among other things, the researchers hope to tell whether transposons move systematically to particular sites, which may have an important bearing on plant evolution.

The jumping event is expected to occur in only one out of every 5,000 to 10,000 plants. For this reason, the experiment must be performed on such a large scale outside a greenhouse.

Heinz Saedler of MPI, the project leader, says that planting must begin by July at the latest if the experiment is to be done this year. But he is "not too optimistic", given the number of federal and Land authorities that might hold it up. **Steven Dickman**

* SPOT: *Deux Yeux Braques sur la Terre*. Bernard Cervelle. Presses du CNRS, Paris; 1989.

Solidarity calls for change

London

POLAND'S multi-viewpoint 'round table' of experts, convened to seek a solution to the country's long-standing economic and social problems and from which has emerged the concession of open elections to one of the two parliamentary chambers, is also agreed on at least one thing; the urgent need for improvements in science and education.

At the opening of the subgroup on science and education last month, statements by the co-chairmen, Education Minister Jacek Fisiak for the government/party side and Professor Henryk Samsonowicz, former rector of Warsaw University, for the Solidarity/opposition wing, turned out to be virtually interchangeable.

Both said that Polish science and higher education have been damaged by long years of financial neglect, low wages and the lack of social prestige for teachers, and the restriction on the universities' rights to decide their own staffing policy, research and teaching plans.

The difference between the two spokesmen was chiefly one of timing: Solidarity wants immediate change, the official side would prefer to go more slowly. But by the final meeting of the subgroup last Saturday, a timetable had been agreed.

Although the round table includes representatives of all strata of Polish society, from Politburo members to clerics, philosophers, steelworkers and private farmers, its main achievement (and one that took several months to negotiate) has been to bring to the official conference table some of the chief figures from the 'democratic opposition' and the Solidarity movement.

Thus the education and science group includes such scholars as Stefan Amsterdamski and Wladyslaw Kunicki-Goldfinger from the Society of Academic Courses ('Flying University') of the late 1970s, Wiktor Kulerski, Solidarity's main spokesman on education during its legal existence (1980-81) and later a leader of underground Solidarity for the Warsaw region, and Wladyslaw Findeisen, who was removed from his post as rector of Warsaw Technical University in 1985 for refusing to discipline students who took part in pro-Solidarity rallies.

Likewise, the medical subgroup was co-chaired by Dr Zofia Kuratowska, whose haematological clinic was closed in 1985 in reprisal for her work among internees during the martial law period. And Janusz Onyszkiewicz, the Warsaw University lecturer in mathematical logic, resumed his former role of chief press spokesman for Solidarity — at least for the duration of the round table.

But the presence of Solidarity at the

round table does not mean that life in Poland is completely back to the *status quo ante* martial law. As Samsonowicz noted, several cases of teachers and lecturers dismissed or penalized on political grounds during the martial law period are still outstanding. Fisiak has accepted a list of these cases "for review" and told a press conference that many other professionals are now being reinstated, there could be no reason why teachers and lecturers should not have the same satisfaction.

This represents a major policy shift. The rationale behind the education ministry's power to intervene against university staffs on political grounds, laid down in the 1985 higher education act, is precisely that teachers and lecturers are not comparable with other kinds of workers

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Lech Walesa counts the achievements of the roundtable talks last week (AP).

because they influence and shape the outlook of the next generation.

The 1985 act, which largely negated the liberalizations of the 'Solidarity' period and which was introduced in the face of concerted protest from the universities, is now itself scheduled for revision. But shortly after the subgroup began work, Fisiak's promise to restore a measure of autonomy to the universities seemed imperilled when student demonstrations in Krakow took a violent and overtly anti-Soviet note. The reforms, warned government press spokesman Jerzy Urban, depended on university authorities realizing that "autonomy does not mean anarchy".

Solidarity spokesmen have nevertheless been urging that if the banned pro-Solidarity Independent Students' Association (NZS) was reinstated, the students would not have to take to the streets to make their grievances known. The round table, Lech Walesa said, needs three legs in order to stand firm. Two legs had been provided by government promises to legalize Solidarity and the private farmers' organization 'Rural Solidarity'. The third 'leg' — legalization of the NZS — was eventually promised, during a meeting last week between Walesa and Interior Minister General Czeslaw Kiszczak.

Vera Rich

Industry co-sponsorship

Paris

YVES Sillard, the former French coordinator of the European EUREKA programme seeking industrial co-sponsorship for competitive product research, thinks the principle could be extended to defence research. Sillard, who recently took over as head of the French Committee on Armaments (DGA), would like to see industry put up venture capital for pre-production studies of new military applications, in the expectation that military approval would be rewarded with government production contracts.

Military research now accounts for more than a third of French government research and development spending (about \$4,000 million). Sillard believes that as European trade barriers fall in 1992, there will be opportunities for transnational collaboration, even if some domains (such as nuclear arms) would not be suitable. Sillard's idea is being studied by the DGA before being put to other European defence agencies.

Peter Coles

INFORMATION TECHNOLOGY

No action on research

London

THE British government last week rejected calls from the House of Commons Trade and Industry Committee for an increase in the number of undergraduate and graduate courses in information technology (IT) in order to tackle an emerging skills shortage.

The committee had said, in a report on IT, that there are 30,000 unfilled vacancies for people with IT skills and that the shortage was worsening. But the government now says, in a policy paper setting out policy on IT, that measures to combat the shortage have already been introduced. A £43-million programme launched four years ago will by 1990 have provided 5,000 extra places on undergraduate and post-graduate courses, it said.

It also rejected the committee's call for increased spending on research and development in IT, saying that £100 million will be spent by the government next year for this purpose. It stressed that the chief responsibility for financing industrially relevant research lies with industry, and rejected the committee's suggestion that the government should encourage industrial investment in IT by identifying a target level of research. It said that the government would not require that investment be disclosed in company accounts, as this would be burdensome to industry.

The committee had called for reinstatement of the post of minister for information technology, on the grounds that since the post was abolished there has been no significant increase in interest in the subject among ministers. But the government says it is neither feasible nor desirable to locate responsibility for all aspects of IT policy in a single department. Christine McGourty

Earthquake risk

SIR—The recent earthquake in Armenia has once again focused attention on the need to adhere to some minimal building code in order to minimize earthquake hazards, especially in developing countries in seismically active zones (*Nature* **336**, 625; 1988). This is the only practicable solution for such countries, where the development of facilities and capabilities for data-acquisition and data-processing to monitor the build-up of strain and to predict its release in earthquakes is still far in the future.

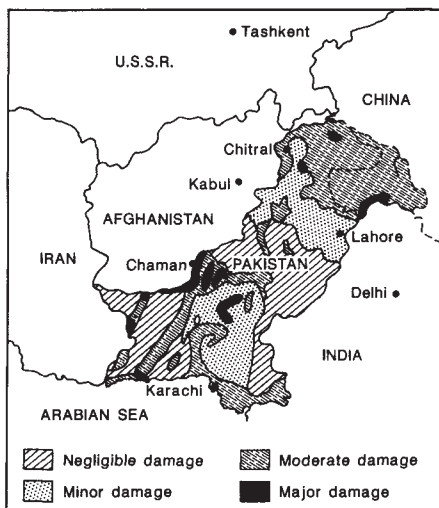
Towards the middle of the next century, the developing countries in Asia with a history of destructive earthquakes will experience a growth of development in communication, energy, irrigation and industry, financed largely by international agencies and executed under the supervision of experts in the countries concerned.

These agencies and experts must make themselves responsible for minimizing natural hazards, of which earthquakes are the most sudden and destructive. They should perhaps specify that a seismic zoning map should be available when a large project is planned.

A seismic zoning map of Pakistan (see below) is being published for the information of international funding agencies and for those involved in structural designs for dams, power and chemical plants and so on in Pakistan.

ABUL FARAH

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This map, constructed from macro-earthquakes data from 1905 to 1979, can be used for risk assessment if one assumes that if ground motion of a certain intensity is experienced once in a certain area, it is likely to be experienced again in that area. Map adapted from Building Code of Pakistan, Ministry of Housing & Works, Islamabad, 1986, pp 11–38 to 52.

Looking-glass land

SIR—Our solution of the molecular structure of foot and mouth disease virus (*Nature* **337**, 709; 1989) involved an intriguing number of coincidental connections with the life and career of Lewis Carroll, the mathematician and author of the Alice in Wonderland books. He was born and spent his early years in Daresbury, the location of the SERC synchrotron source where the X-ray diffraction data were collected. He later moved to Oxford to teach mathematics and it is there that the data were analysed and the structure of the virus solved. Carroll also owned a house in Guildford, which is close to Pirbright, where the virus was grown and crystallized. The final link was provided by the representation of the virus structure featured on the cover of *Nature*. This picture is as the virus would have been seen by Alice through the looking-glass — it was printed as the mirror image of the true structural organization of the particle.

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Aluminium limits

SIR—It has been known since 1983 that a blood serum level of aluminium in excess of 0.1 mg per l presents a significant risk of neurological damage¹, yet the permissible level of aluminium in European Communities domestic water remains at 0.2 mg per l. Are we not expecting too much from the European kidney?

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1. Savory, J. & Berlin, A. *Annals of clinic. Lab. Sci.* 444–451 (1983).

Improbability

SIR—Respondents to Bruce Denness's query (*Nature* **336**, 614; 1988 and **337**, 498; 1989) are surely correct that scientists need not be too much concerned with far-fetched theories that are fabulously contrived to explain phenomena. But the fantastical context of the discussion should not be permitted to obscure a serious, but related, scientific problem.

One reason to reject the spontaneous or wilful creation of the Universe in the recent past is that we have a good explanation that hinges on improbabilities of the recent past on the even more improbable

(in the sense of lower entropy) less-recent past. But in a Big-Bang cosmology, this particular buck can be passed back only so far. How did the earliest times come to be so improbable? Roger Penrose has estimated¹ that the original conditions of our Universe, if generated randomly, had a likelihood comparable to 1 part in 10 to the power of 10¹²³. It is not clear that such a staggeringly improbable initial condition is any easier to arrange than admittedly preposterous, direct fabrication of a recent state of the Universe replete with falsified history.

WILLIAM ECKHARDT

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1. Penrose, R. "Time-Asymmetry and Quantum Gravity" in *Quantum Gravity 2*. Isham, Penrose and Sciama (Oxford, 1981).

Anthropic principle

SIR—I was surprised to learn in the slightly misleading report from M. Abramowicz and G. F. R. Ellis (*Nature* **337**, 411–412; 1989) that a feature of the Venice Conference on cosmology and philosophy (18–19 November 1988) had been my abandonment of the final anthropic principle. In fact, my talk was concerned primarily with other subjects. However, the documented discussion following other speakers reveals that the scientific basis of this proposal was presented and defended. It is particularly interesting in view of recent ideas about information processing and complexity. For a truer picture, I refer the interested reader to the conference proceedings which will be published by Cambridge University Press under the editorship of U. Curi.

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Retraction test

SIR—I agree with John Maddox (*Nature* **338**, 13; 1989) on the importance of full retraction by an author who discovers nontrivial mistakes in his or her previously published work. It is a litmus test of scientific integrity.

To any researcher hesitant about publishing a retraction when necessary, I would say that I can immediately think of colleagues whose reputations went up in my estimation when they published retractions.

I was horrified to read that some funding bodies apply the opposite criterion. This must be stopped.

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Risks from ionizing radiation

R.H. Clarke and T.R.E. Southwood

For the public in the United Kingdom — and the United States — natural radon is a far greater radiation hazard than are emissions from nuclear installations.

RADON indoors now accounts for nearly half of the average UK population exposure to ionizing radiation. We believe that the extent of the variation in exposure to radon in the United Kingdom and elsewhere and its magnitude in relation to exposures from other natural and artificial sources needs to be more widely recognized. Such information should influence attitudes to risk and the allocation of resources for programmes of radiological protection. We cannot help but observe the large investment being made in the United Kingdom to reduce doses from the Sellafield nuclear reprocessing plant¹, in comparison with that being made to reduce exposure from indoor exposure to radon².

The National Radiological Protection Board monitors the exposure of the UK population to all sources of ionizing radiation. We have just completed our latest assessment³, which is based as far as possible on measurements of exposure from natural background radiation, medical diagnostic procedures, fallout of radionuclides and occupations involving use of radiation. Some exposures are too low to be measured, in which case we calculate doses using environmental and dosimetric models, such as consumer products and the average dose from effluents from nuclear power stations.

We estimate that the average exposure within the United Kingdom is now 2.5 millisieverts (mSv) per year compared with just under 2.2 mSv per year when we last reported⁴. The increase is principally due to a re-assessment of exposure to indoor radon gas as a result of our national survey⁵ of the levels and the re-evaluation of the dose-intake relationship for radon and its daughters. The contributions of various sources of radiation in 1988 are listed in Table 1 in descending order of importance, natural sources being identified and the doses from 1984 being given for comparison. There have been reductions in occupational exposure and from nuclear effluents in the period since our 1984 report, but these are small compared with the increased estimate for radon.

In the United States, the national average exposure to ionizing radiation has been estimated recently to be 3.6 mSv, more than 1.4 times the UK average⁶. (The breakdown of components of dose in the United States is also shown in Table 1.) Medical exposures to individuals are

higher in the United States than in the United Kingdom, as are doses from consumer products and other miscellaneous sources. It is clear that higher average radon exposures in the United States account for most of the difference from the UK figures, although the US distribution is not yet well known.

National averages are useful for broad

TABLE 1 Contributions to average radiation exposure (mSv)

UK		Source	USA
1988	1984		1987
1.2	0.7	*Radon from earth materials (98 per cent from indoor exposure)	2.0†
0.35	0.40	*Terrestrial γ -rays	0.28
0.30	0.37	*Ingested natural radionuclides	0.39
0.30	0.25	Medical exposures	0.53
0.25	0.30	*Cosmic rays	0.27
0.10	0.10	*Thoron from earth materials	—
0.01	0.01	Fallout (including Chernobyl)	0.0006
0.01	0.01	Miscellaneous sources	0.05-0.13
0.005	0.009	Occupational exposure	0.009
0.001	0.002	Radioactive effluent discharges	0.0005
TOTAL			TOTAL
2.5	2.2		3.6

* Natural sources

† Includes thoron

comparisons, but any average conceals a wide range. The dose received by single individuals may depart markedly from the average, depending on their mode of life and geographical location. Therefore, in the figure, we contrast doses received by different groups in the UK population with the national average figures. The area of each pie diagram is proportional to the total dose received, and the slices represent the percentages from the various sources.

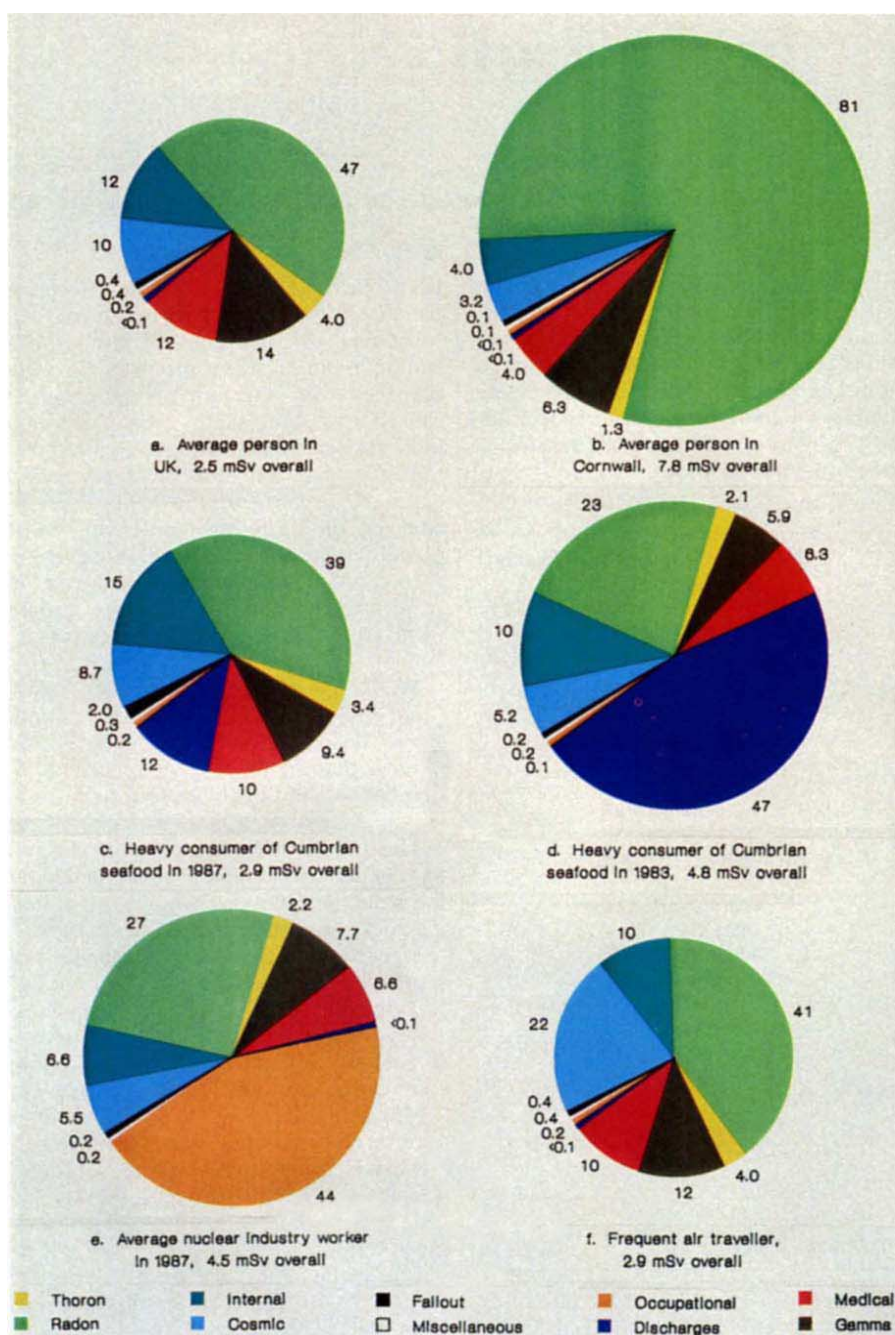
In general, the highest doses in the United Kingdom arise in Cornwall and Devon in the south-west of England. By comparison with the UK average (*a* in the figure), the average dose received in Cornwall (*b*) is slightly more than three times higher (7.8 compared with 2.5 mSv), principally because of the nature of the local geology. The terrestrial γ -radiation is slightly higher, but the main contribution

to the increase arises from the effects of the inhalation indoors of radon and its daughters (81 per cent of the total). Radon in houses arises primarily through seepage from the underlying ground, caused by the slight under-pressure indoors. Better insulation reduces ventilation in houses and thus increases radon exposure because of the lower rate of changes of fresh air⁷. But apparently obvious remedies, such as the use of an extractor fan, may cause an increase in under-pressure and draw more radon from the ground, making the situation worse³.

We know of parts of Cornwall, and some other areas of the United Kingdom, where the exposure of individuals can rise to more than 20 mSv in a year when more than 90 per cent of the dose results from indoor radon⁷.

The public and the media in Britain have generally been more concerned with the safety of artificial sources of radionuclides, in particular the effects of discharges to the Irish Sea from the Sellafield reprocessing plant in Cumbria in the north of England. Those receiving the highest doses in this region are the avid consumers of seafoods collected off the Cumbrian coast near the plant (*c* in the figure), whose dose is increased to 0.33 mSv compared with the UK average of 0.001 mSv⁸. Nevertheless, the total exposure of these people is 2.9 mSv per year, not much higher than the national average, and much less than the value of 4.8 mSv per year (*d* in the figure) which we reported in 1984 (ref. 4). These lower doses result from the reduced discharges of radionuclides from the nuclear reprocessing plant at Sellafield achieved since 1984.

We also note the increase in natural internal exposure associated with high shellfish consumption (*c* in the figure) from the increased intake of natural α -emitting radionuclides (mainly polonium-210) that are concentrated by shellfish. This increase, which is unrelated to any man-made source of radionuclides, can raise the annual internal dose of an individual by 0.14 mSv (from 0.30 to 0.44 mSv), just under half the additional dose now received by those most exposed to the Sellafield discharges. Because of the pattern of rainfall in May 1986, people living in Cumbria (and in other parts of western Britain) are still exposed to slightly higher than average doses of radiation because of residual fallout from the



Variations of annual radiation dose in the United Kingdom compared with the average. Pie areas are proportional to dose and percentage segments to sources.

reactor accident at Chernobyl (0.05 mSv in 1987 compared with the average of 0.01 mSv).

The effect of occupational exposure on the pattern of total dose is shown in *e* in the figure, which is the dose for a typical UK nuclear industry worker in 1987. Occupational exposure does, of course, vary from one individual to another, as well as for the same individual at different times of life. (The average dose of 2 mSv is obtained from about 20,000 reactor workers receiving doses averaging less than 1 mSv per year together with more than 5,000 reprocessing-plant workers averaging about 5 mSv per year¹.)

Other features of modern life have a

significant effect on dose received — because of the reduction in the screening effect of the atmosphere with height, for example, air travel increases exposure to cosmic radiation². Frequent air travellers who, in a year, fly for 100 hours (about five subsonic return flights between Britain and the west coast of North America, or a weekly flight between London and Scotland or a nearby European country) would increase their dose from that source from 0.25 to 0.65 mSv per year. This would lead to a total dose of 2.9 mSv per year as shown in *f* in the figure — the same dose as received by those most exposed to discharges from Sellafield.

Our new survey³ shows that radon is the greatest single source of radiation to the population. Radon and its daughters are α -emitters, which are more biologically damaging than β - and γ -radiations. Is there a real risk of lung cancer from radon exposure in the home? There are data from studies on uranium miners who have been chronically exposed to radon and its daughters⁴, and it is encouraging that our estimate for the risks of lung exposure derived from the Japanese atom-bomb survivors⁵ is close to the figure for the uranium miners. Experiments on rodents exposed to radon result in similar risks, and confirm a linear relationship between risk and dose⁶.

Depending on the exact risk factors applied, radon exposure in the United Kingdom might be responsible for 6 per cent or more of the annual incidence of lung cancer (41,000). We recommend an action level for radon exposure in homes corresponding to 20 mSv in a year¹², and similar action levels are being considered in Sweden and the EEC¹³. In the United States, action is recommended at 7.5 mSv in a year¹⁴, and there is legislative pressure for abatement as well as for programmes of intervention and research¹⁵. Our advice in 1987¹² was that lifetime exposure to radon at its action level would give rise to about a 2 per cent chance of an individual dying of lung cancer. It is now clear that this figure has to be revised substantially to at least 5 per cent, and that action to reduce the risks from radon are even more urgently required. □

R.H. Clarke is Director and Sir Richard Southwood Chairman of the National Radiological Protection Board, Chilton, Didcot, Oxford OX11 0RQ, UK.

- House of Commons First Report from the Environment Committee, Session 1985–86, *Radioactive Waste* Vol. 1, LXI (HMSO, London, 1986).
- Department of the Environment *The Householders Guide to Radon* (HMSO, London, 1988).
- Hughes, J.S., Shaw, K.B. & O'Riordan, M.C. *The Radiation Exposure of the UK Population 1988 Review* NRPB-R227 (NRPB, Chilton, 1988).
- Hughes, J.S. & Roberts, G.C. *The Radiation Exposure of the UK Population 1984 Review* NRPB-R173 (NRPB, Chilton, 1984).
- Exposure to Radon Daughters in Dwellings* NRPB-GS6 (NRPB, Chilton, 1987).
- Ionizing Radiation Exposure of the Population of the United States* Report No. 93 (NCRP, Washington, DC, 1987).
- Wrixon, A.D. et al. *Natural Radiation Exposure in UK Dwellings* NRPB-R190 (NRPB, Chilton, 1988).
- Hunt, G.J. *Radioactivity in Surface and Coastal Waters of the British Isles, 1987* (MAFF, Lowestoft, 1988).
- BEIR IV: *Health Risks of Radon and Other Internally Deposited Alpha-Emitters* (National Academy Press, Washington, DC, 1988).
- Stather, J.W. et al. *Health Effects Models Developed from the 1988 UNSCEAR Report* NRPB-R226 (NRPB, Chilton, 1988).
- Cross, F.T. in *Radon and its Decay Products in Indoor Air* (eds Nazaroff, W.W. & Nero, A.V., Wiley, New York, 1988).
- Exposure to Radon Daughters in Dwellings* ASP.10 (NRPB, Chilton, 1987).
- Commission of the European Communities *Radon in Indoor Air* EUR11917EN (CEC, Luxembourg, 1988).
- US Environmental Protection Agency *A Citizen's Guide to Radon* OPA-86-004 (USEPA, Washington, DC, 1986).
- US Department of Energy *Radon Research Program, Financial Year 1988* (Office of Health and Environmental Research, USDOE, Washington, DC, 1988).

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Space Telescope ready at last

The long delay in launching the Hubble Space Telescope, caused by the Challenger disaster, has given the Space Telescope Science Institute time to develop strategy, and to win the respect of the community.

Baltimore

WHEN the Hubble Space Telescope (HST) is finally launched, maybe in December, the most sophisticated automatic telescope yet built will be in a 90-minute orbit 300 miles above the Earth, but its users will be left firmly on the ground. The Space Telescope Science Institute (STScI) here will be the nearest they can get to it, which may be regarded as a way of carrying to its extreme the growing belief among those who operate major telescopes that the presence of live astronomers is often an inconvenience, sometimes even a hazard.

As at observatories everywhere, users will submit proposals which will be assessed for scientific merit and their particular suitability for the instrument they intend to use. Successful proposals will be worked up in detail, with the help of staff astronomers at the STScI, and interleaved with all the other successful proposals to make the most efficient use of available observing time and instrumentation.

But HST has to fulfil expectations beyond the relatively straightforward hopes of scientists: it (or more particularly the people who operate it) must prove that the money and anxiety spent on it, especially during the enforced hiatus caused by the Challenger disaster, have not been in vain. The US Congress, and US taxpayers generally, will be looking eagerly over the users' shoulders for surprises and novelties. Some astronomers may regard this element of showmanship as an intrusion; fortunately, the director of STScI, Riccardo Giacconi, is not one of them. But what spectacles to provide?

The charter of STScI contains the useful provision that its purpose is to "conduct" an observing programme on the Space Telescope. This gives the director the freedom to act either as the passive arbitrator of proposals or as the dictator of what observations should be made. The more pharaonic extreme will be valuable in the early days.

Even if the Space Telescope is launched as intended during December this year (and difficulties with the imminent launch of Discovery cast some doubt on this), it will not be ready to spring instantly to life. The first six months have been allocated to commissioning the telescope and its various instruments. This period can be stretched by one more month, but after that STScI intends to gain control of the Space Telescope because, as one astronomer said, the engineers would carry on

commissioning the instrument for the whole of its fifteen-year lifetime, given the chance.

During commissioning, trial observations will be made with the telescope's six instruments (two cameras, two spectrographs, a photometer and a fine-guidance system for astrometry). Some data and images will therefore be coming down to Earth before observations officially begin. Will these do for Congress?

STScI is working under a written obligation from NASA that any such 'first light' images used for promotional or other purposes must specifically not have any scientific interest. The supposed danger is that if, for example, *Nature* were to publish on its cover sometime next January an unprecedentedly fine picture of a nearby globular cluster, in which the central stars could be made out individually instead of blurring together, then eager astronomers around the world would get out their plastic rulers and start counting stars, uncovering long-held secrets about the internal dynamics of dense stellar systems and depriving whichever group of astronomers had the first proposal to do the same thing of their chance for glory. STScI is thus faced with the task of producing first images which are both spectacular and uninteresting, a riddle not yet solved.

Once the observing programme begins in earnest, scheduling tactics will be more conventional, but some special difficulties remain. For the Space Telescope, the cycle of day and night is replaced by the 90-minute orbital period, and although an object near the North or South pole can be observed continuously, those parts of the sky are generally empty of interesting objects: the galactic plane is close to the plane of the Solar System and the Earth's Equator, which means that most observing will have to be done in the 45-minute portions of time when the Earth is not in the way. On top of that, the Space Telescope must not be allowed to point at the Moon or the Sun, which would be fatal to some of the instrumentation.

The speed of the orbit combined with the telescope's lack of speed in slewing across the sky (its direction can be changed only a little faster than the minute hand moves on a clock) combine to make efficient scheduling difficult. During the launch delay, STScI has developed scheduling algorithms to maximize the time that observations are being made.

Switching from one instrument to

another is a headache, involving as it does choosing between six detectors, selecting one of about fifty filters, changing filters and detectors during an observing run, and perhaps running two instruments simultaneously. STScI will be pleased to be making observations one-third of the time.

Deciding what observations to make is the final part of STScI's strategic puzzle. Ultimately, one of the Space Telescope's greatest virtues may lie in its ability to perform long-term projects, such as dissecting and cataloguing distant clusters of galaxies or searching for infrared emission from nearby subdwarf stars.

The deadline for the first round of proposals passed last October, and almost 600 proposals from 1,500 astronomers in 30 countries have been received. About 15 per cent of the observing time is guaranteed to investigators from the European Space Agency, which has provided the Faint Object Camera and the solar panels for HST, but the 15 per cent target is intended only as a long-term average, and there will be no lawsuits if the proportion during the first year or so is a little amiss.

Observing time is oversubscribed by about seven to one for this first period, which has allowed Giacconi and his staff to exercise their discretionary powers to slant observations in chosen directions without sacrificing quality. Many of the programmes that STScI favours are of cosmological importance: finding Cepheid variable stars at the distance of the Virgo cluster of galaxies, for example, would bypass several rungs of the cosmological distance ladder and could at a stroke reduce our ignorance of the Hubble constant and the age of the Universe.

Giacconi has understood from the outset that the ability of STScI to impose, to some extent, its designs on the rest of the astronomical community depends on the standing of his institute. STScI scientists win about 20 per cent of the observing time at Kitt Peak National Observatory.

It is a trite comment now that the years of delay to HST's launch have been beneficial, allowing the instruments to be improved, a satisfactory number of guide stars to be catalogued, and scheduling problems to be overcome. But more important is that the delay has allowed STScI to become an astronomical institution of the first rank whereas, had HST been launched on time, it might have been merely a service station. **David Lindley**

Revving up gene expression

Michael R. Green and Maria L. Zapp

THE expression of eukaryotic protein-coding genes can be regulated at a variety of levels from transcription to translation. It has been largely assumed, however, that the transport of the fully processed messenger RNA from the nucleus to the cytoplasm (mRNA nuclear export) is a constitutive step. Studies of the human immunodeficiency virus (HIV) reported by Malim *et al.* on page 254 of this issue¹ suggest that this assumption is no longer tenable, and may stimulate the quest for cellular proteins that regulate the export of RNAs from the nucleus.

All replication-competent retroviruses, including HIV and the somewhat similar human T-cell leukaemia virus (HTLV-I), have three essential structural proteins: the group-specific antigen, the envelope protein, and the reverse transcriptase, encoded respectively by the *gag*, *env* and *pol* genes. HIV and HTLV-I are, however, souped-up retroviruses, which also encode unique proteins that regulate viral gene expression. Two HIV regulatory proteins are encoded by the *tat* and *rev* genes, and the corresponding HTLV-I proteins by the *tax* and *rex* genes² (see Fig. 1).

The HIV Tat and HTLV-I Tax proteins act at the transcriptional level to increase

the intron-containing *env* and *gag-pol* mRNAs. One way Rev and Rex could effect this increase is by specifically inhibiting splicing. This would raise the amount of unspliced *gag-pol* mRNA and singly spliced *env* mRNA at the expense of the *tat/rev* mRNA, whose production requires two splicing events. Alternatively, Rev and Rex could act by specifically accelerating the nuclear export of the *env* and *gag-pol* mRNAs. (These two possibilities are not mutually exclusive as accelerated nuclear export is itself a mechanism for inhibiting splicing, a point we shall return to.)

Malim *et al.*¹ present three lines of evidence, which together argue that Rev does not directly inhibit splicing. The first set of experiments asks what is the RNA sequence(s) required for the effect of Rev (Rev-responsive region; RRE)? The existence of an RRE is implied by the fact that the viral *env* mRNA, but not a cellular mRNA precursor (pre-mRNA) such as insulin, is responsive to Rev. To test whether the RRE corresponds to a viral splicing signal, chimaeric genes were constructed in which each of the known splicing signals of the viral *env* RNA was replaced by the equivalent insulin pre-mRNA signal. All of the chimaeric pre-

mRNAs were still responsive to Rev, indicating that no viral splicing signal is uniquely required for Rev action. Next, Malim *et al.* mapped the RRE to a portion of the *env* coding sequence that is spliced out of the *tat/rev* mRNA (intron 2), and showed that it functions following translocation to several positions within intron 2, and even when moved to the exon.

The ability of the RRE

to work independently of its position within the RNA, and particularly within the exon, makes it unlikely that Rev acts at the level of splicing.

In a second set of experiments, Malim *et al.* measured the ratio of unspliced-to-spliced *env* RNA in nuclei and cytoplasm. If Rev directly inhibits splicing, the increased ratio of unspliced-to-spliced *env* RNA in the cytoplasm should be paralleled in the nucleus. However, Rev has little effect on the ratio of unspliced-to-spliced *env* RNA in the nucleus, arguing that Rev does not directly affect either splicing or nuclear stability. (Note, however, that this conclusion relies upon assumptions regarding the rate-limiting

step in splicing, and cytoplasmic mRNA production, and potential inter-relationships between splicing and export; see below.) Finally, Malim *et al.* tested the splicing inhibition model by asking whether Rev can act on a pre-mRNA rendered unsplicible by removal of the 5' splice site. They find that Rev increases the cytoplasmic concentration of this unsplicible RNA, a result more compatible with the nuclear export model than the splicing inhibition model.

Comparable experiments have not yet been performed with the HTLV-I Rex protein, but it is probable that Rev and Rex act similarly. The HTLV-I Rex protein can functionally substitute for the HIV Rev protein³, an intriguing observation in the light of the fact that these two proteins lack amino-acid similarity.

Although Rev may not directly inhibit splicing, by accelerating nuclear export Rev inhibits splicing indirectly: export separates the pre-mRNA from the nuclear-localized splicing factors. Splicing occurs in the nucleus following assembly of the pre-mRNA into a large complex, the spliceosome, which contains multiple small nuclear ribonucleoproteins (snRNPs). The spliceosome may also serve to retain RNAs in the nucleus by, for example, preventing the RNA from productively interacting with the nuclear pore, the normal avenue of egress^{4,5}. In this sense, spliceosome assembly and nuclear export are competing processes (Fig. 2), a notion supported by recent experiments in yeast in which mutations that prevented spliceosome assembly facilitated nuclear export⁶.

Upon splicing, the mRNA is released from the spliceosome, which remains primarily associated with the intron. The mRNA, but not the excised intron, is then free to be exported. This 'spliceosome-retention' model can explain the selectivity of RNA export: for a typical cellular pre-mRNA, the spliced mRNA product is exported, whereas the unspliced pre-mRNA and the other product of the splicing reaction, the excised intron, are retained in the nucleus.

Retroviral pre-mRNAs are spliced notoriously inefficiently, perhaps because of specific inhibitory *cis*-acting elements^{7,8}. This inefficiency is probably critical to the

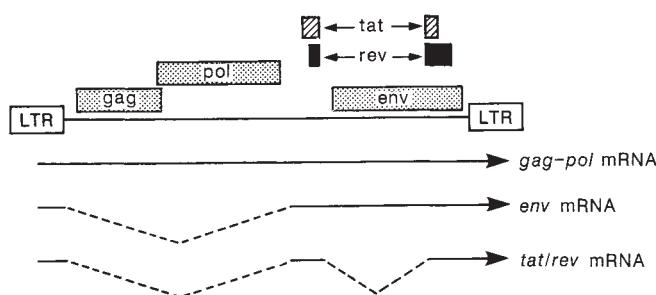


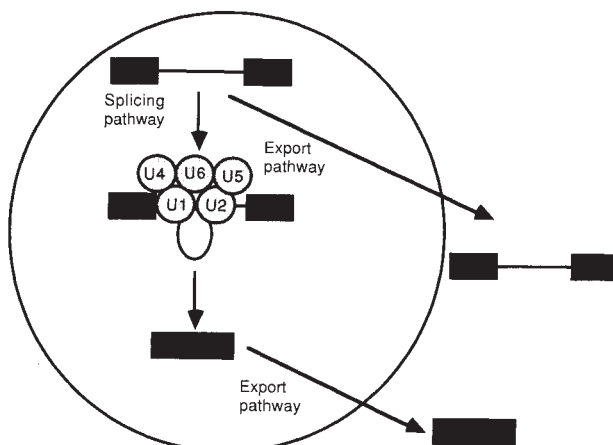
FIG. 1 A simplified version of HIV gene expression. The regulatory proteins of HIV (and HTLV-I) are expressed from largely overlapping reading frames. The HIV proviral genome is shown bracketed by the two LTRs. The viral protein-coding regions are shown above. A schematic representation of the splicing patterns of the viral mRNAs is shown below. Exons, solid lines; introns, dashed lines.

synthesis of both the structural and regulatory viral proteins. The HIV Rev and HTLV-I Rex proteins, however, selectively increase the synthesis of the structural proteins later in infection, thereby maximizing the production of viral particles. The basis for this selective action lies in a second unique feature of HIV and HTLV-I: both viral genomes contain at least two introns. Both introns are present in the *gag-pol* mRNA, whereas one intron is removed to produce the *env* mRNA, and both are spliced out of the mRNA(s) encoding the regulatory proteins (Fig. 1).

Rev and Rex augment the production of viral structural polypeptides by increasing the cytoplasmic concentration of

1. Malim, M.H., Hauber, J., Le, S.-Y., Maizel, J.V. & Cullen, B.R. *Nature* **338**, 254-257 (1989).
2. Gallo, R., Wong-Staal, F., Montagnier, L., Haseltine, W.A. & Yoshida, M. *Nature* **333**, 504 (1988).
3. Rimsky, L. *et al.* *Nature* **335**, 738-740 (1988).
4. Dworkin, S.I. & Feldherr, C.M. *J. Cell Biol.* **106**, 575-584 (1988).
5. Featherstone, C., Darby, M.K. & Gerace, L. *J. Cell Biol.* **107**, 1289-1297 (1988).
6. Legrain, P. & Rosbash, M. *Cell* (in the press).
7. Katz, R.A., Kotler, M. & Skalka, A.M. *J. Virol.* **62**, 2686-2695 (1988).
8. Arrigo, S. & Beemon, K. *Molec. Cell. Biol.* **8**, 4858 (1988).
9. Beyer, A.L. & Osheim, Y.N. *Genes Dev.* **2**, 754 (1988).
10. Zasloff, M., Rosenberg, M. & Santos, T. *Nature* **300**, 81-84 (1982).
11. Zasloff, M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 6436 (1983).
12. Tobian, J.A., Drinkard, L. & Zasloff, M. *Cell* **43**, 415 (1985).
13. Siomi, H. *et al.* *Cell* **55**, 197-209 (1988).

FIG. 2 Splicing and export pathways in the eukaryotic cell nucleus, showing potential processing pathways for a pre-mRNA in the nucleus. The pre-mRNA is shown with boxes representing exons, and lines representing the intron. In a splicing pathway, the pre-mRNA is assembled into a spliceosome. In the spliceosomal complex, U1, U2, U4, U5 and U6 snRNPs are shown as circles. Some of the snRNP–snRNP contacts in the spliceosomal complex are arbitrarily drawn.



propagation of the virus, because incompletely spliced and unspliced RNAs are required to encode structural proteins and to serve as genomic RNA. Conceivably, Rev may be able to work only upon inefficiently spliced pre-mRNAs. For example, Rev may act before spliceosome assembly, and efficiently spliced pre-mRNAs are probably assembled rapidly into spliceosomes. In fact, electron microscopy has shown that the spliceosome assembles immediately on the nascent transcripts of some cellular genes⁹.

A more detailed understanding of the mechanism of Rev (and presumably Rex) may be hampered by our limited knowledge of mRNA nuclear export. For example, we do not even know whether mRNAs require a specific export signal, or whether, if not encumbered by a spliceosome, they are exported by default. The existence of single-base substitutions that

can prevent export of transfer RNAs^{10–12}, argues that at least some RNAs harbour specific export signals.

How might Rev accelerate the rate of export? Recent immunofluorescence experiments may provide a clue. Surprisingly, the HTLV-I Rex protein is localized in the nucleolus rather than the nucleoplasm or nuclear envelope¹³, perhaps implicating the nucleolus or a nucleolar constituent in mRNA nuclear export.

Although Rev might just be a quirk of HIV, studies of eukaryotic viruses have often provided the first glimpses into cellular regulatory mechanisms. If history repeats itself, Rev and Rex are the harbingers of cellular regulatory proteins waiting to be discovered. □

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MATERIALS SCIENCE

Fractal dimension and fracture

Robert W. Cahn

How rough is a fracture surface? This question is in the domain of the craft of fractography, which seeks to elucidate the mechanisms of fracture by studying the topography of a broken object. Somewhat akin to phrenology, some sceptics might say. Nevertheless, in two new studies, Mecholsky, with Mackin¹ and with Passoja and Feinberg-Ringel², shows that it may be possible to scale the fracture toughness of fairly brittle materials by just such an exercise in fractographic analysis, exploiting the concept of fractals.

Fractals were invented — or at any rate clearly formalized — by Mandelbrot^{3,4}. The term refers to any boundary or surface which remains self-similar as the scale of examination is magnified. Figure 1 gives an example of such a boundary. For this shape, a 'Koch island', drawn to be always of constant area, the length of the perimeter is increased by a factor of 1.5 from one drawing to the next, if the length of the ruler unit used to measure it is

decreased by half, and the image resolution grows by a factor of four. If the logarithm of the total perimeter length is plotted against the logarithm of the length of the ruler unit (the resolution), a straight line or 'Richardson plot' is obtained, the slope of which was treated by Mandelbrot as a fractional, fractal, dimension: for a boundary as in Fig. 1, this dimension is between 1 and 2 — in this case, 1.5.

Similarly, a rough surface which is self-similar has a dimension between 2 and 3. To obtain this fractal dimension, it is usual either to make serial sections perpendicular to the surface ('vertical' sections) and examine the shape of the section boundary, or to section parallel to the sur-



FIG. 1 A Koch island with a perimeter of fractal dimension 1.5 (from ref. 5).

face and examine the perimeters of the 'islands' and 'lakes' which are in effect contours of such 'horizontal' sections.

The original concept of fractals was restricted to self-similar outlines only, but more recently the concept has been extended to more ordinary boundaries and surfaces of varying roughness; thus, Fig. 2 shows four outlines generated by Russ⁵ by a rather involved computer algorithm, with four different fractal dimensions. In such cases, the corresponding Richardson plot has a straight central part with curved regions at either end. (Such shapes should really be called 'pseudo-fractals', but that term does not appear to have been used.) Stereologists still disagree about the appositeness of the horizontal section approach⁶, but in a recent study of fracture surfaces of ceramics, Mecholsky and Passoja⁷ found that the horizontal and vertical methods gave closely similar fractal dimensions.

The studies of Mecholsky and colleagues^{1,2,7} on the relation between fractals and fracture were stimulated by two apparently independent initiatives. One was a fractographic study by Mandelbrot *et al.*⁸ of broken specimens of maraging steel. The impact toughness of 300-grade maraging steel in various states of heat-treatment increases steadily as the fractal dimension of the fracture surface decreases towards 2 (it becomes steadily smoother)⁸. It is apposite that Mandelbrot should have been involved in this early study because, as he says, he coined the term fractal "in explicit cognizance of the fact that the irregularities found in fractal sets are often strikingly reminiscent of fracture surfaces in metals (though not, for example, in glass)". Although he does not say so, one should not lose sight of the fact that the word fraction has the same Latin root as fracture, and fractals have fractional dimensions.

The second study was by Beauchamp and Purdy⁹, who examined the changes in fracture toughness and fracture morphology of chert (flint) as it is heat-treated at progressively higher temperatures. It turns out that chert heated to 500 °C is considerably less tough than untreated material; the heated chert also has a much smoother surface. (This implies that heated chert is easier to chip and shape into tools, and indeed there is archaeological evidence that chert was commonly heated in prehistoric times.) Heating coarsens the exceedingly fine grain of the chert and also apparently enhances the strength of the intergranular bond: unheated chert fractures along the boundaries of the fine grains, heated chert breaks transgranularly. But the unheated material is nevertheless tougher because fracture is constrained to follow a very tortuous path.

Beauchamp and Purdy did not explicitly invoke the fractal concept in their study.

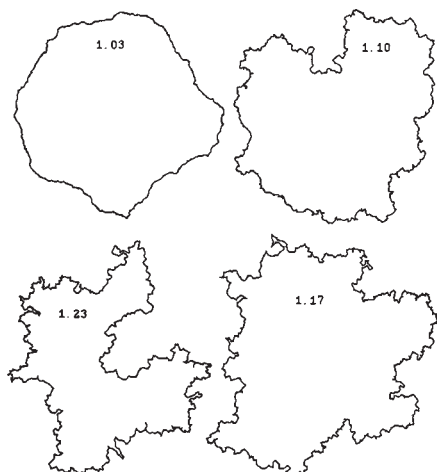


FIG. 2 A random fractal outline (from ref. 5).

but in the new work^{1,2} by Mecholsky and co-workers, their original samples are subjected to a proper fractal analysis, using both horizontal and vertical methods of analysis. It is quite clear that the fracture toughness, K_{IC} , of chert increases as the fractal dimension increases — as the fracture surface becomes rougher. This is the exact converse of what was found for maraging steel. (The relationship for chert is in the form of a linear log-log plot of K_{IC} against fractal dimension.)

The authors also indicate, by means of a figure relating the fractal dimension of the fracture surface to fracture toughness for several ceramics (oxides, silicates, chalcogenides) that this form of relationship is universal for such highly brittle materials, though the results bunch into several distinct material groups rather than falling along a single master line. This research is itself a continuation of earlier work⁷.

We thus have the intriguing fact that the relationship between fracture roughness (fractal dimension) and fracture toughness is diametrically opposite for ductile fracture (maraging steel) and for brittle fracture (chert and polycrystalline ceramics). For chert, where fracture is governed by the spreading of cracks, the toughening influence of factors making for rough fractures has been qualitatively explained. For ductile fracture, which can take place by the progressive creation, growth and merging of minute voids, the relationship is clearly different. Mandelbrot *et al.*⁸ seek to interpret their findings in terms of percolation concepts applied to the filaments remaining intact between voids, but it must be said that their argument is not easy to follow. It is to be hoped that in future work, Mecholsky and colleagues will address the piquant distinctions between ductile and brittle fracture which these studies have uncovered. □

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1. Mecholsky, J.J. & Mackin, T.J. *J. Mater. Sci. Lett.* **7**, 1145–1147 (1988).
2. Mecholsky, J.J., Passoja, D.E. & Feinberg-Ringel, K.S. *J. Am. Ceram. Soc.* **72**, 60–75 (1989).
3. Mandelbrot, B.B. *The Fractal Geometry of Nature* (Freeman, New York, 1982).
4. Gleick, J. *Chaos: Making a New Science* 94–118 (Heinemann, London, 1988).
5. Russ, J.C. in *Encyclopedia of Materials Science and Engineering Suppl.* Vol. 1 (ed. Cahn, R.W.) 169–175 (Pergamon, Oxford, 1988).
6. Underwood, E.E. & Banerji, K. *Mater. Sci. Engng.* **80**, 1–14 (1986).
7. Mecholsky, J.J. & Passoja, D.E. in *Fractal Aspects of Materials* 117–119 (Mater. Res. Soc., Pittsburgh, 1986).
8. Mandelbrot, B.B., Passoja, D.E. & Paullay, A.J. *Nature* **308**, 721–722 (1984).
9. Beauchamp, E.K. & Purdy, B.A. *J. Mater. Sci.* **21**, 1963–1966 (1986).

MICROBIOLOGY

Bacteria that thrive in toluene

Howard Goldfine

TOLUENE is widely used in the laboratory to permeabilize and kill bacteria. Thus, the isolation of a bacterial strain described by Inoue and Horikoshi on page 264 of this issue¹, which not only resists the toxic actions of toluene, but grows luxuriantly in the presence of emulsions containing up to 50 per cent of this solvent, represents a significant achievement. In a rich medium, Inoue and Horikoshi find that growth in the presence of toluene is about two-thirds as rapid as that obtained in its absence. It should, however, immediately be noted that the newly isolated strain only tolerates toluene rather than using it as an energy source.

The organism, identified as a strain of the gram-negative aerobic *Pseudomonas putida*, an organism known for its protean degradative capabilities, was isolated after screening 750 soil samples collected in Japan. The only hint concerning the site

of its isolation is that the soil was obtained from Kyushu, Japan's large southern island. It is not clear whether these soils are contaminated with organic solvents.

The isolation of organisms resistant to aromatic hydrocarbons and other industrial products has implications for biodegradation and biotransformation. The annual global production of benzene and toluene each exceeded 10⁹ gallons in 1980, and these materials are likely to be found in soils and sediments². The availability of organisms that can withstand the toxic effects of these organic solvents and participate in their degradation or transformation is clearly desirable. *P. putida* contains chromosomal genes encoding the enzymes involved in the degradation of aromatic compounds by the so-called ortho cleavage pathway, and strains have been isolated that contain the common TOL plasmids encoding the genes for the

enzymes of the catechol meta cleavage pathway³. It would seem to be just a short step to bring together these degradative resistance characteristics. The isolated strain is also resistant to styrene, *p*-xylene, ethylbenzene, cyclohexane, *o*-dichlorobenzene, propylbenzene and various alkanes up to isooctane, thus there is wide scope for producing both solvent-tolerant and solvent-degrading strains. The marriage of these two desirable traits may be less than harmonious, however. The alterations in the cells of the toluene-tolerant strain that make growth in solvents possible could shield the cell from these solvents so perfectly that little penetration to the locations of degradative enzymes would be possible.

The authors of the paper in this issue, one of whom is a member of the Superbugs Project of the Research Development Corporation of Japan, have begun a genetic analysis of the solvent resistance of *P. putida*. They have isolated a series of mutants of their new strain that are not resistant to toluene, but retain resistance to solvents of lower polarity in the order toluene > *p*-xylene > cyclohexane > hexane.

By examining the partitioning index of these and other substances in a standard octanol:water mixture, they can predict accurately the resistance of these mutant strains to other compounds in the polarity scale. In addition to mutant strains of *P. putida*, they can order other microorganisms in a similar hierarchy of solvent-tolerance, in which growth in a solvent of a given polarity indicates tolerance to solvents of lower polarity. Genetic analysis combined with biochemical and morphological studies should provide useful insights into the nature of solvent tolerance in these bacteria.

The outer membrane of gram-negative bacteria is important in determining the penetration of hydrophobic molecules through cell membranes, and consequently in determining the resistance of these bacteria to dyes, detergents, bile salts and hydrophobic antibiotics. Mutations in the structure of the lipopolysaccharide of the outer membrane can profoundly affect permeability of these molecules, but these effects are complicated by concomitant changes in the amounts of specialized pore-forming, outer-membrane proteins⁴. Studies of the outer membranes of these solvent-resistant strains of *P. putida* may be rewarding.

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1. Inoue, A. & Horikoshi, K. *Nature* **338**, 264–266 (1989).
2. Gibson, D.T. & Subramanian, V. in *Microbial Degradation of Organic Compounds* (ed. Gibson, D.T.) 181–252 (Dekker, New York, 1984).
3. Bayly, R.C. & Barbour, M.G. in *Microbial Degradation of Organic Compounds* (ed. Gibson, D.T.) 253–294 (Dekker, New York, 1984).
4. Nikaido, H. & Vaara, M. *Microbiol. Rev.* **49**, 1–32 (1985).

Networks from mutants

Dennis Bray and Juri Vasiliev

THOSE of us who earn a living from the cytoskeleton feel a little threatened. We had always asserted (rather too dogmatically, as it now seems) that actin and actin-binding proteins were the motor by which animal cells crawl from one location to another. And yet André *et al.* now describe in the current issue of the *Journal of Cell Biology*¹ the third mutant strain of *Dictyostelium* which lacks a major actin-binding protein and nevertheless shows normal motility and chemotaxis. The ground begins to shake beneath our feet! Could it be that, like those primitive people who believed that when the wind blows it is because the trees are stirring the air, we have the wrong end of the stick? Is it possible that cell locomotion is driven not by the cytoskeleton but by lipids, or something equally improbable?

The amoeboid form of *Dictyostelium discoideum* crawls in a very similar fashion to other animal cells and also shows a chemotactic response to distant sources of cyclic AMP. Unlike neutrophils and fibroblasts, however, these cells are haploid and therefore particularly suitable for genetic manipulation. Gerisch and his colleagues² at the Max Planck Institute in Martinsried in 1986 took advantage of this combination of virtues by isolating a mutant deficient in the actin crosslinking protein α -actinin. The group's more recent study³ of this mutant, using four monoclonal antibodies, confirms that α -actinin is almost completely absent; nevertheless, the mutant's movements, chemotaxis and ability to 'cap' and 'patch' surface proteins are unimpaired.

An indication that this might be a general phenomenon came in two papers^{4,5} published in the same issue of *Science* last year describing life without myosin. As related in a News and Views article at the time⁶, strains of *D. discoideum* were generated that lacked normal high-molecular-weight myosin (myosin-2). In one case, expression of the myosin gene was severely depressed through the introduction of antisense RNA; in the other, homologous recombination was used to substitute a myosin lacking most of its tail for the normal myosin. It is something of a relief to relate that these myosin-less cells are not entirely normal: they have an altered morphology and are severely defective in their ability to carry out cytokinesis. But they can crawl and they can direct their locomotion up a gradient of cyclic AMP.

The point has now been driven home in the new work of Gerisch's group¹, in which André *et al.* describe a mutant strain of *Dictyostelium* lacking severin — an actin-filament fragmenting protein

related to gelsolin and villin. The cells grow at the usual rate, forming normal fruiting bodies and viable spores. By many criteria, such as speed of movement, rate of turning and precision of chemotactic orientation, their motility is unimpaired.

A literal interpretation of these findings, based on conventional genetic arguments, would be that α -actinin, myosin and severin are not involved in cell locomotion. But this conclusion is at variance with much circumstantial evidence linking actin-based systems to the generation of motile structures in the cell. An alternative explanation is that these proteins could be involved in cell locomotion but their function could be duplicated or 'guaranteed' (as Schleicher *et al.* put it⁷) by more than a single protein. There is indeed substantial overlap in the activity of actin-binding proteins *in vitro* and more than one way to crosslink, fragment or even to move actin filaments. But why should there be such extensive 'fail-safe' guarantees for cell locomotion when

other, more essential cellular functions such as DNA synthesis are not thus underwritten?

The explanation that we favour — which, if correct, has implications that extend beyond the area of cell motility — is that actin-based motility has some of the properties of the parallel distributed processes used in artificial intelligence (see Anderson's News and Views article⁷).

That is, at every stage between the detection of environmental cues by receptors on the cell surface to the generation of cell movement, there is extensive overlapping redundancy. Cell surface receptors change the levels of second messengers such as calcium ions and phosphatidyl inositol biphosphate; second messengers influence the activity of actin-binding proteins, either directly or through protein kinases. At each successive stage, the 'signal' generated by a specific agonist becomes distributed more widely; it spreads down parallel pathways and converges with various combinations of signals from other receptors on to different target proteins.

The ten or more actin-binding proteins in a cell would form one layer of this net-

Paternity of dunnocks by DNA fingerprinting

IMAGE
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THE hedge sparrow or dunnock (*Prunella modularis*) is a small, insect-feeding passerine bird belonging to the accentor family. It is common throughout the British Isles, inhabiting bushy places where nesting sites are plentiful and is a common sight in suburban parks and gardens. The species is unusual in having a variable mating system, a fact which has attracted considerable attention from behavioural ecologists. Mating may involve monogamy (one male, one female), polygyny (a male with two females), co-operative polyandry, where two males mate with a single female and may help to feed her young, and polygynandry, where two males share two or more females. On page 249 of this issue, T. Burke and collaborators report the successful application of the technique of DNA fingerprinting to link observations of mating behaviour and parental care of dunnocks in a well-studied population in a botanical garden in Cambridge, UK with precise measurements of reproductive success. They conclude that males do not discriminate between their own young and those of another male in multiply-sired broods, but feed offspring in relation to their access to the female during the mating period, which is a good predictor of paternity.

Rory Howlett

W. B. Carr

in a set of different motile structures, each of which contains other proteins that perform similar functions. In this view, cell locomotion depends on the combined activities of actin-binding proteins as a whole (if Gerisch and his colleagues mutate all actin-binding proteins, the cell would grind to a halt). But it is a corporate exercise in which individual proteins have a range of functions that overlap the functions of other proteins. Removal of a single protein by mutation will not have a single identifiable phenotype but cause a 'graceful degradation' (a term used in parallel distributed processing) over a range of motile functions.

The analogy with parallel distributed processing networks may seem far-fetched, but it is no more than a recognition of the extensive interconnection in cell signals that is known to exist. The consequences of such overlaps have, simply, been most thoroughly explored in the case of computer networks used to simulate cognitive functions⁸.

One important feature that has been found in such networks is that they are resistant to damage: as already mentioned, this feature is shared by the machinery of cell movement. Another is that individual units within the network come to repre-

sent regularities in the input: we might suppose, for example, that a rise in activity of a particular protein kinase represents all those sets of environmental conditions that signify 'round up' or 'divide'. Third, and of greater significance to evolution, is that parallel distributed networks are highly flexible in performance. Each type of cell movement could be driven by a specific 'blend' of actin-binding proteins ("take three ounces of actin; add three cups of filamin and two of severin; mix with a tablespoon of myosin and stir. . ."). And there are many more possible combinations of actin-binding proteins than there are individual proteins: an argument familiar to developmental biologists. □

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1. André, E. et al. *J. Cell Biol.* **108**, 985-995 (1989).
2. Wallraff, E. et al. *EMBO J.* **5**, 61-67 (1986).
3. Schleicher, M. et al. *J. Cell Sci.* **90**, 59-71 (1988).
4. Knecht, D.A. & Loomis, W.F. *Science* **236**, 1081-1086 (1987).
5. De Lozanne, A. & Spudis, J.A. *Science* **236**, 1086-1091 (1987).
6. Sheetz, M. *Nature* **331**, 212 (1988).
7. Anderson, J.A. *Nature* **322**, 406-407 (1986).
8. Rumelhart, D.E. & McClelland, J.L. *Parallel Distributed Processing* (MIT, Cambridge, Massachusetts, 1987).

ARCHAEOASTRONOMY

Eclipse seen at ancient Ugarit

Christopher B. F. Walker

THE engraved tablet shown below is the only record of an arguably astronomical nature from the second millennium BC apart from those of Babylonia. Recovered from the site of Ugarit, an ancient city on the Mediterranean coast of Syria, it has been interpreted by Sawyer and Stephenson^{1,2} as a report of a solar eclipse. On page 238 of this issue³, de Jong and van Soldt deduce a new dating of this eclipse, to 1223 BC, basing their argument on archaeological considerations and a reassessment of the text. Their new dating is of great interest to those trying to calculate the change in speed of the Earth's rotation, but is inevitably speculative.

The publication and study of ancient

and mediaeval astronomical records in recent decades has considerably increased the time base over which astronomical phenomena can be considered. The records contain references to eclipses, occultations, comets, sunspots and supernovae⁴. Babylonian⁵ and Chinese⁶ records have lately contributed to the study of the history of Halley's comet. The Babylonian 'astronomical diaries' from the period 400-50 BC⁷ also contain records of market prices and weather reports which may eventually be useful for the reconstruction of economic and climatic patterns. Usable data of the same type from earlier than the seventh century BC are very rare.

The most important single group of

observations has been those of the first and last visibility of Venus recorded during the reign of the Babylonian king Ammisaduqa (1702-1682 BC). Reconsideration of these in the light of later Babylonian data on the *arcus visionis* of the planet have led to a revision of the calculated date of the observations and consequently of the whole chronology of the Old Babylonian period⁸. The Ugaritic tablet is the only other arguably datable astronomical observation outside Babylonia from the second millennium BC.

In assessing the strength of the claims made¹⁻³ for the Ugaritic tablet, one must take into account the fact that this is, if correctly identified, the sole surviving astronomical observation written in the Ugaritic language. The only related material preserved in the palace archives of Ugarit are two fragments described as almanacs and an astrological text. Whereas Babylonian astronomical terminology, including the terminology of eclipses, is well known, we have no information on Ugaritic terminology. At first sight the text refers to an event occurring at sunset. Its interpretation as a non-technical description of a solar eclipse was suggested by Sawyer and Stephenson^{1,2} on the basis of similar circumlocutory descriptions of eclipses ranging from China in the thirteenth century BC to Germany in the twelfth century AD. Corroboration of the interpretation depends on the planetary and calendrical evidence.

The identification of the Ugaritic god's name Ršp with Mars and the proximity of Mars to the eclipsed Sun are critical to de Jong and van Soldt's analysis. The Ugaritic names for the planets are otherwise unknown, and the identification depends on the twice-attested equation of Ršp with the Mesopotamian god Nergal and on a single Assyrian astrological omen text of the seventh century BC in which Mars, elsewhere always named Sabtanu, is unusually given the name Nergal. The normal usage in Mesopotamia was to give the specific names of the planets in observational references, and to introduce the names of associated deities only in astrological explanations. The use of the name Ršp in the Ugaritic text would contrast with this pattern.

As de Jong and van Soldt make clear, their interpretation of the text as an eclipse report is also dependent on the assumption that a calendar of Egyptian

Obverse side of the Ugaritic tablet KTU 1.78, presently in the Museum of Antiquities in Damascus. Arguably this describes a solar eclipse, dated by de Jong and van Soldt in this issue³ to have occurred in 1223 BC. (Courtesy of D. Pardee.)

IMAGE
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Mission Archéologique de Ras Shamra/Ugarit

1. Sawyer, J. F. A. & Stephenson, F. R. *Bull. Sch. Oriental Afr. Stud.* **33**, 467-489 (1970).
2. Stephenson, F. R. *Nature* **228**, 651-652 (1970).
3. de Jong, T. & van Soldt, W. H., *Nature* **338**, 238-240 (1989).
4. Stephenson, F. R. & Clark, D. H. *Applications of Early Astronomical Records* (Hilger, Bristol, 1978).
5. Stephenson, F. R., Yau, K. K. C. & Hunger, H. *Nature* **326**, 587-592 (1985).
6. Stephenson, F. R. & Yau, K. K. C. *J. Br. Interplanet. Soc.* **38**, 195-216 (1985).
7. Sachs, A. J. & Hunger, H. *Astronomical diaries and related texts from Babylonia* Vol. 1 (Austrian Acad. Sci., Vienna, 1988).
8. Huber, P. *Astronomical Dating of Babylon I and Ur III* (Undena, Malibu, California, 1982).

type was in use. Until now there has been nothing to demonstrate whether Ugarit followed the Egyptian or the Babylonian pattern. Thus the present assumption, while perfectly plausible, will remain speculative until supporting evidence becomes available.

Given the paucity of contemporary material it is perhaps unlikely that we will ever find more explicit astronomical reports from Ugarit, and we have to make

the best use of what we have. De Jong and van Soldt's work is certainly an advance on the earlier discussion. But the limitations of the evidence are such that at present their interpretation can best be regarded as a plausible hypothesis. To an Assyriologist it would seem to be a precarious basis for scientific arguments. □

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PICORNAVIRUSES

Finding the receptors

Stephen C. Harrison

NEW insights and new puzzles about how RNA viruses recognize their receptors arise from the structure of foot-and-mouth disease virus, published three weeks ago in *Nature*¹, and from the identification of the receptors for human rhinoviruses and for poliovirus, reported this month in *Cell*^{2,3}. The structure of the foot-and-mouth disease virus also provides a plausible explanation for the efficacy of a particular peptide vaccine.

Like other picornaviruses, foot-and-mouth disease virus (FMDV) is composed of 60 copies each of four polypeptides (VP1–4), arranged with icosahedral symmetry. Although not obviously related in sequence, VP1, 2 and 3 have similar folded structures, based on an eight-stranded β -barrel that I have elsewhere⁴ proposed be called an RVC domain (for RNA virus capsid, where it was discovered) — see figure. The β -sheet framework is similar in the three subunits, but the loops and amino- and carboxy-terminal extensions vary considerably. VP4 is, in effect, part of the amino-terminal extension of VP2, from which it is cleaved during assembly. The human rhinovirus 14 (HRV14)⁶ and poliovirus⁷ structures show that the loops between framework elements dominate the surface of the virus and provide the important antigenic determinants.

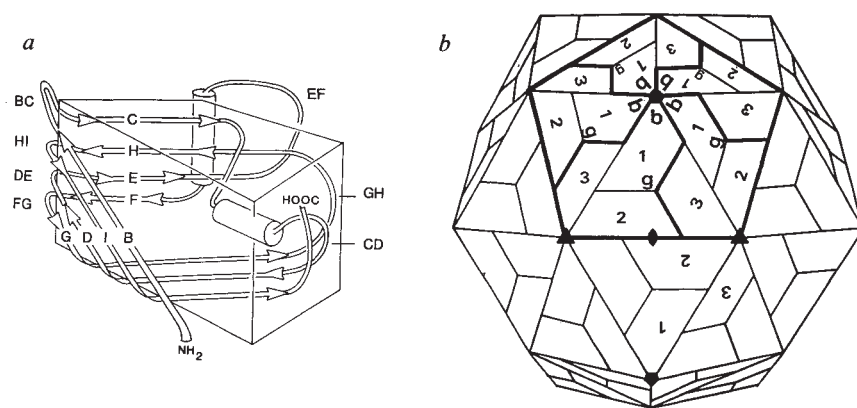
In HRV14 and in poliovirus, VP1 contains particularly prominent BC and GH loops at opposite ends of the wedge-like barrel. These loops create a concave surface, and the packing of five copies of VP1 around the 5-fold symmetry axis means that these concavities join to form a roughly circular groove (see figure caption). This feature in HRV14 has been called the 'canyon' by Rossmann and colleagues⁸. These authors proposed that it might be the locus of receptor binding and noted that it would be inaccessible to antibodies. Colonno and colleagues have obtained evidence that the HRV14 canyon is its receptor site, by generation of site-directed mutants in infectious complementary DNA clones⁹.

Several viruses show antigenic variation that permits escape from neutralization by

antibodies present in a host previously exposed to a parent strain. Conserved receptor sites cannot undergo this variation. Must they be inaccessible to avoid neutralization by antibodies against their conserved structure? Colman has observed that a conserved receptor site need not compromise potential escape mutants, provided that the site is smaller than the typical 'footprint' of an antibody⁹. Variation at the periphery of the site can prevent neutralization without interfering with receptor interactions. Indeed, the influenza virus receptor site is a small, sialic acid-binding pocket of conserved amino acids surrounded by residues that vary between recurrent influenza epidem-

ics¹⁰. The FMDV structure seems to show another kind of attachment site with similar properties. Several lines of evidence point to participation of the GH loop of VP1 in receptor binding¹¹. Relatively short peptides with sequences from this loop block attachment of virus to cells¹¹. They contain an evolutionarily conserved Arg-Gly-Asp sequence, which is surrounded by residues that vary from strain to strain. Not only does the GH loop project from the viral surface, it is also sufficiently disordered that it does not appear in the electron-density map. Thus, the presumed binding site in FMDV appears to be prominent, not recessed, but it is smaller than an immunoglobulin 'footprint' and embedded in a variable surround.

Potent vaccines can be made from peptides having sequences in the disordered GH loop of VP1¹². The unusual success of these peptide vaccines can probably now be explained. Like a peptide in solution, the loop has no unique, folded conformation. A given antibody molecule that has been raised against a peptide antigen will be complementary to that peptide in a particular conformation. The corresponding sequence in the flexible loop can adopt the required conformation with little cost in free energy. Moreover, if different specificities in a serum recognize different conformations of the same peptide epitope, all will bind well to the virion.



a, The folded arrangement of the polypeptide chain in an RVC domain, as found in icosahedral, positive-strand RNA viruses. The framework strands of the eight-stranded β -barrel are denoted by letters B–I. The intervening loops and terminal extensions vary significantly among RNA viral proteins, but the size and shape of the framework is relatively constant. The upper surface of the domain in this diagram faces the outside of the particle when packed in the capsid of an RNA virus. The structure of tumour necrosis factor (TNF), a non-viral protein with a similar folded conformation, is described by Jones *et al.* on page 225 of this issue²⁵. TNF looks very similar to a simple RVC domain, but its packing in a trimer involves different interfaces from those used by subunits of RNA viruses. Barrels of similar topology, but of rather different size and shape, are found in other viral and non-viral proteins. **b**, The packing of RVC domains in an icosahedral viral shell. Some of the copies of VP1, 2 and 3 are labelled. There are 60 copies of each in the virus. VP4, cleaved from the amino terminus of VP2, is internal. The letters 'b' and 'g' show positions of the BC and GH loops in VP1. In FMDV, the BC loop of VP1 is truncated and the GH loop is disordered. The virus particle therefore has 'dimples' on its 5-fold symmetry axes and a flexible protrusion near the positions marked 'g' (and their symmetry equivalents). In HRV14 and in poliovirus, the BC and GH loops of VP1 are both well-ordered and prominent. The cavity between them forms part of a groove or canyon that circulates around the 5-fold axes (roughly joining the positions marked '1' in the upper part of the diagram).

Neutralization of picornaviruses by antibodies is believed to require some degree of 'crosslinking' between subunits, perhaps to inhibit an essential expansion or concerted rearrangement of proteins in the capsid^{13,14}. The flexibility of the GH loop in VP1 of FMDV could facilitate such crosslinking. Correlations between disorder and response to anti-peptide antisera have been noticed before^{15,16}. The GH loop of FMDV is a particularly striking example and the first to be associated with a successful vaccine.

Cells of many types contain adhesion proteins — collectively called integrins — that recognize the Arg-Gly-Asp sequence in proteins in the extracellular matrix¹⁷. The inhibition of FMDV attachment by peptides containing Arg-Gly-Asp receptors suggests that one or more of the integrins is the FMDV receptor. The definitive identification of receptors for two other picornaviruses — human rhinovirus and poliovirus — implicates other kinds of adhesion proteins as viral binding targets. Two families of human rhinoviruses have been identified, recognizing distinct receptors¹⁸. The main group binds to a protein of relative molecular mass 90,000¹⁹ that has now been identified in two laboratories as intercellular adhesion molecule-1 (ICAM-1), a surface protein found on many kinds of cells²⁰. This molecule mediates leukocyte adhesion by binding to a protein called lymphocyte function-associated molecule-1 (LFA-1), a homologue of the integrins²⁰. ICAM-1 itself is a member of the so-called immunoglobulin superfamily — proteins with domains similar to domains of the immunoglobulin molecule. It contains five concatenated immunoglobulin-like domains, a transmembrane segment and a short cytoplasmic tail^{21,22}. The poliovirus receptor, also just identified, has a rather similar structure, with three rather than five immunoglobulin-like domains, but it

seems to correspond to a previously unidentified member of the family⁴.

Receptor specificity is generally regarded as a major determinant of cell and tissue tropism as well as of virus host range. In this regard, the distribution and expression of ICAM-1 and of the poliovirus receptor raise real questions. Both appear to be expressed in tissues that do not normally support growth of the virus that recognizes them²⁻⁴. Indeed, messenger RNA encoding the poliovirus receptor can be detected in cells to which virus does not even attach⁴. Yet the new papers show that when polio or HRV receptors are expressed in transfected mouse L cells, they confer ability to bind virus²⁻⁴.

Adhesion molecules such as ICAM-1 and the integrins might seem to be odd candidates for viral receptors. Poliovirus and HRV 14 are believed to enter cells by the receptor-mediated endocytosis pathway²³. Rapid endocytosis is not expected to be a property of matrix and intercellular-attachment proteins, however, and how these viruses are chaperoned into

coated pits and endosomes now becomes a significant puzzle. Are adhesion molecules endocytosed when clustered (requiring polyvalency of viral attachment)? Or is attachment only the first in a more elaborate chain of events? In other words, as the groups studying it point out, ICAM-1 is a highly suitable receptor for rhinoviruses. Its expression on epithelial and endothelial cells is significantly induced by soluble mediators of inflammation²⁴. Therefore, a local virus infection may indirectly enhance the abundance of nearby receptors, through mechanisms of the host inflammatory response, or interfere with that response, by blocking ICAM-1. The more general notion, that in viral pathogenesis a virus-receptor interaction may do more than merely mediate attachment, is strengthened by these observations. □

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ENZYME CHEMISTRY

Designer catalytic antibodies

Robin J. Leatherbarrow

ENZYMES are well known to offer many advantages to the synthetic chemist. They often exhibit great specificity for the reaction catalysed, and work under mild conditions of temperature and pH in aqueous solution. The number of possible synthetic reactions, however, far exceeds the specificities of currently characterized enzymes, so there is considerable interest in producing enzymes with novel specificities by artificial means. Two routes have been used — protein engineering to alter the specificities of enzymes (ref. 1, for example); and the use of antibody molecules, which bind specific ligands (haptens) but which do not normally carry out chemical reactions. By clever design of the hapten it is possible to generate antibodies which catalyse specific reactions in the same way as an enzyme. On page 269 of this issue², Shokat *et al.* describe a new route to generate such catalytic antibodies.

Production of catalytic antibodies (abzymes) was first described in 1986 in simultaneous publications by the groups of Lerner³ and of Schultz⁴. Their approach was based on two principles: that enzymes work by binding the transition state of a reaction better than the ground state; and that antibodies which bind specifically to any small molecule can be produced by coupling this molecule to a protein carrier and immunizing experimental animals. Lerner's and Schultz's groups reasoned that antibodies that were produced to bind transition-state analogues (molecules

designed to mimic the shape and electronic configuration of the transition state) would function as enzymes towards the substrate of the reaction. Since then there has been considerable success in producing abzymes catalysing acyl-transfer reactions, carbon-carbon bond forming, and carbon-carbon bond cleaving reactions using these techniques (see ref. 2 and references therein).

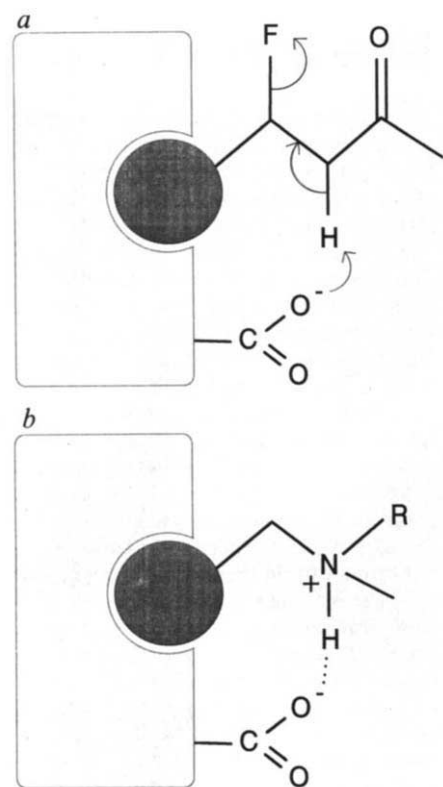
Although the transition-state analogue approach has been successful, examination of natural enzymes shows that while some mechanisms involve exclusively transition-state stabilization⁵, it is very common for groups within the active site to participate in the reaction. Acid/base catalysis involving proton donation or abstraction occurs particularly frequently. The problem addressed by Shokat *et al.*² is how such groups can be placed in the active site of an antibody with the precision required to generate catalytic activity. The reaction the authors chose was a β -elimination, reasoning that catalysis would be obtained by positioning a carboxyl group on the abzyme to abstract a proton. The desired reaction is shown in *a* in the figure. The abzyme needs a binding site for the main part of the substrate, and a carboxyl group which reacts as shown.

To produce this abzyme the authors immunized mice with a hapten containing a positively charged group, which they hoped would result in antibodies having a complementary negatively charged carboxyl group in the correct position to

1. Acharya, R. *et al.* *Nature* **337**, 709–716 (1989).
2. Greve, J.M. *et al.* *Cell* **56**, 839–847 (1989).
3. Staunton, D.E. *et al.* *Cell* **56**, 849–853 (1989).
4. Mendelsohn, C.L. *et al.* *Cell* **56**, 855–865 (1989).
5. Harrison, S.C. in *Concepts in Viral Pathogenesis* (eds Notkins, A.L. & Oldstone, M.B.A.) (Springer, New York, 1989).
6. Rossmann, M.G. *et al.* *Nature* **317**, 145–153 (1985).
7. Hogle, J.M. *et al.* *Science* **229**, 1358–1365 (1985).
8. Colonna, R.J. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **85**, 5449–5453 (1988).
9. Colman, P.M. *et al.* *Nature* **303**, 41–44 (1983).
10. Weis, W. *et al.* *Nature* **333**, 426–431 (1988).
11. Fox, G. *et al.* *J. gen. Virol.* (in the press).
12. Bittle, J.L. *et al.* *Nature* **298**, 30–32 (1982).
13. Ruecker, R. in *Virology* (eds Fields, B.N. *et al.*) **705-738** (Raven, New York, 1985).
14. Emini, E.A. *et al.* *J. Virol.* **48**, 547–550 (1983).
15. Tainer, J.A. *et al.* *Nature* **312**, 127–134 (1984).
16. Westhof, E. *et al.* *Nature* **311**, 123–126 (1984).
17. Ruoslahti, E. *et al.* *Rev. Biochem.* **57**, 375–413 (1988).
18. Abraham, G. & Colonna, R.J. *J. Virol.* **51**, 340–345 (1984).
19. Tomassini, J.E. & Colonna, R.J. *J. Virol.* **58**, 290–295 (1986).
20. Marlin, S.D. & Springer, T.A. *Cell* **51**, 813–819 (1987).
21. Simmons, D. *Nature* **331**, 624–627 (1988).
22. Staunton, D.E. *et al.* *Cell* **52**, 925–933 (1988).
23. Marsh, M. & Helenius, A. *Adv. Virus. Res.* (in the press).
24. Dustin, M. *et al.* *J. Immunol.* **137**, 245–254 (1986).
25. Jones, E.Y., Stuart, D.I. & Walker, N.P.C. *Nature* **338**, 225–228 (1989).

Emerging stability and chaos

Andrea Milani



a, Schematic representation of the reaction to be catalysed. The abzyme (light grey) requires a binding site for the substrate, together with a carboxylate group placed to carry out proton abstraction. *b*, The hapten used to generate the abzyme. A positively charged group is present so that the resulting antibody will tend to have a complementary negatively charged group involved in binding.

react (*b* in the figure). Of six monoclonal antibodies isolated, four accelerate the rate of β -elimination. This is a very high success rate, and suggests that the approach will be valuable in other reactions.

Given the mechanism of the reaction shown in *a*, one can speculate on the effect of introducing other charged groups into the abzyme. A positive charge near the fluoro group may be expected to enhance the polarization of the C-F bond and increase the rate of reaction. An additional carboxyl group (negatively charged) could be built into the hapten for this purpose, in an attempt to generate an appropriate abzyme. Depending upon the chirality of this carboxyl hapten, it may be possible to create abzymes specific for one or other of the enantiomeric forms of the fluoro substrate, and so confer stereospecificity on the reaction. □

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1. Wilks, H.M. *et al.* *Science* **242**, 1541–1544 (1988).
2. Shokat, K.M., Leumann, C.J., Sugawara, R. & Schultz, P.G. *Nature* **338**, 269–271 (1989).
3. Tramantano, A., Janda, K. & Lerner, R.A. *Science* **234**, 1566–1570 (1986).
4. Pollack, S., Jacobs, J. & Schultz, P.G. *Science* **234**, 1570–1573 (1986).
5. Leatherbarrow, R.J., Fersht, A.R. & Winter, G. *Proc. natn. Acad. Sci. U.S.A.* **82**, 7840–7844 (1985).

HAVE the planets been on more or less the same orbits, with only quasiperiodic variations, since the birth of the Solar System? This problem of the stability of the Solar System has been investigated for more than 200 years. The problem becomes even more difficult when one asks whether it is possible to compute the orbits of the planets for any time span, or whether there is a predictability horizon¹ beyond which there is no way reliably to compute where the planets will be (or were)? It now seems that the second question will be the first to be answered fully. J. Laskar reports on page 237 of this issue² that the orbits of the inner planets are chaotic: any two orbits with nearby initial conditions diverge exponentially over timescales of 5 million years. Thus any computation spanning more than a few tens of millions of years (much less than the age of the Solar System) gives results that do not reliably apply to nearby orbits, and hence may not apply at all to the real Solar System whose initial conditions are derived from observations of finite accuracy.

Our ability to compute the long-term orbits of the planets has leapt forwards over the past 5 years, mostly because of the availability of supercomputers. The project LONGSTOP brought together several research groups (including my own) to compute the orbits of the outer planets (from Jupiter to Pluto) for 100 million years³. The Digital Orrery, a parallel computer specifically designed and built to solve *N*-body problems, has recently increased this limit to 845 million years⁴.

Laskar uses a 'conventional' supercomputer, but exploits the dedicated computer algebra processors developed at the Bureau des Longitudes in Paris to perform analytical averaging of the equations of motion⁵; this permits the orbits of all the large planets, including the fast-moving Mercury, to be followed for 200 million years. When these figures are compared with the age of the Solar System (4.5×10^9 years), the solution of the problem of the stability of the Solar System might appear close — only one order of magnitude further away. But if the orbits are chaotic, the problem cannot be solved by computing just one orbit for the required time span.

In analytical theories of the planetary orbits a series representation of the solution is constructed, and so-called small divisors occur, as a result of the presence of a dense set of resonances between the fundamental frequencies. A resonance occurs when the ratio between two frequencies, or their combinations, is pre-

cisely the ratio of two integers. Even if the main resonances (with simple ratios such as 3:2) are avoided, the minor ones are always close. As a result, the number of terms that have to be taken into account grows too fast for such a theory to achieve the accuracy which would be needed, even with the most powerful computer algebra systems. This difficulty was known to Poincaré⁶, who showed that any perturbation theory constructed to apply to a set of given initial conditions must give divergent series. Thus the use of supercomputers to perform numerical integrations is not a concession to fashion but the only possible way forward.

The next difficulty is encountered when the results of such numerical integrations become available. Although the series resulting from a purely analytical theory are divergent, there are solutions to the *N*-body problem that can be represented by means of a convergent Fourier series, namely the 'conditionally periodic' solutions found by Kolmogorov, Arnold and Moser (see ref. 7). In most of these there are few fundamental lines and a wealth of combination lines with rapidly decreasing amplitude.

If the solution of the initial-condition problem used to model the Solar System belongs to this class, then the numerically computed solution can be fitted to a conditionally periodic solution. When this technique of the synthetic theory³ was used for the major outer planets, the LONGSTOP team⁴ found that the fit was very poor. The behaviour seems not to be 'conditionally periodic', but rather consistent with a solution chaotically wandering in a thin region of the phase space. For the inner planets, Laskar⁵ has found that a conditionally periodic model is impossible to fit, suggesting a quite large chaotic region.

To some specialists of dynamics, the evidence that an orbit is not a conditionally periodic solution is as good as proving that it is chaotic. Unfortunately, there is no rigorous proof that every orbit must be either conditionally periodic or chaotic, or a case of escape/collision, although there is no evidence for other possibilities at present. Direct proof of chaotic motion for some large planet is also needed. This can be obtained by computing two initially nearby orbits and monitoring the rate of increase of their separation; if the rate is exponential with time, the orbit is chaotic and the coefficient inside the exponential is the (maximum) Liapunov exponent. This was first achieved for the orbit of Pluto by the Digital Orrery team, which recently reported that two nearby Plutos diverge with a Liapunov exponent of 1 per

20 million years; moreover Pluto appears to have a continuous spectrum¹.

Laskar finds an even shorter timescale for the exponential separation of the orbits of the inner planets². In principle, if one planet is in a chaotic orbit, then they all are. But whatever Pluto, Mercury and Mars do, the large outer planets are so little perturbed by them that in practice chaotic effects on the larger planets should occur with negligible amplitude. Thus we do not yet have direct evidence of, for example, Jupiter being in a significantly chaotic orbit, although this is expected to be the case, possibly with a somewhat smaller Liapunov exponent. It should be stressed that the precise values of the Liapunov exponents are extremely sensitive to small changes in the initial conditions, the planetary masses and the model used in the computation; thus the exact values might change as a result of future work. But presuming that the order of magnitude of the Liapunov exponents is correct as currently calculated, it is clear that the orbits of all the planets cannot be reliably computed for a time span as long as the age of the Solar System.

Chaos, however, does not necessarily imply that the system is unstable, that individual orbits will change drastically. Although instability might occur, the timescale over which this will happen is unknown. If the solutions are chaotic, then any statement of the behaviour of the planetary orbits must be of a probabilistic nature, and has to be investigated by computing many solutions with different initial conditions. Although this cannot yet be done, there is a preliminary task that is within reach of our present computing capability, namely to understand the qualitative features of these chaotic motions.

The interesting point is that in systems such as the Solar System — those that are only slightly perturbed from being integrable (analytically solvable) — chaos does not arise at random. Rather, it arises because of resonances, and the region of phase space sampled is shaped by those resonances. An investigation of the resonances in the Solar System should thus permit an approximation of the minimum size of the chaotic region and hence allow the prediction of the likelihood and extent of instability. The computation of the maximum size of the chaotic region is not yet possible: transitions from one resonance to another can occur, even if the two do not overlap, in exponentially long time spans which cannot be calculated except for simple cases.

This resonance analysis is now in progress for the two examples of proven chaotic motion described above — Pluto and the inner planets. An interesting feature seems to be emerging, namely the ubiquitous nature of secular resonance. This feature can be defined as a resonance

in which the longitudes of the planets — with periods between a few months and a few hundred years — do not appear. Because the perihelia and nodes revolve with frequencies, denoted by g_k and s_k , respectively ($k = 1, \dots, 9$), corresponding to periods of tens of thousands of years, a combination frequency can be considered nearly resonant if its period is many millions of years.

Pluto is locked in three different resonances, one involving the longitudes of Pluto and Neptune; another an irregularity of the perihelion of Pluto; and a third, secular resonance recently discovered in our LONGSTOP data (*Icarus*, submitted). All these resonances appear to result in regular librations, however, and not to disrupt the stability of the orbit of Pluto. Another small divisor which might be relevant is $(g_9 - g_8) - 3(s_8 - s_9)$; this combination of the fundamental frequencies is essentially zero in the Digital Orrery solution, and significantly different from zero in the LONGSTOP solution. The cause of this discrepancy is the source of some controversy between the two teams, but clearly very small changes used in the computation, for example to the planetary masses, can result in displacement either inside or outside a resonance with a very small associated chaotic region. For the other outer planets the smallest divisor is $2(g_5 - g_7) + (s_7 - s_8)$, and again there is a suspicion of different behaviour in the different solutions so far computed. In his most recent, as yet unpublished, data, Laskar finds that the divisor $2(g_1 - g_3) + (s_3 - s_4)$ is the main cause of the large Liapunov exponent found for the inner planets. The similarity between these secular resonances is remarkable.

For the moment, we are left with the enigma of an orbit of Pluto locked in so many resonances, chaotic, and still ostensibly stable over timescales of hundreds of millions of years. As for the inner planets, they seem to be even more strongly chaotic, and nevertheless they are still here. The problem is now to understand how chaotic orbits can be stable for timescales much longer than the inverse of the Liapunov exponent. The results from the current generation of long-term orbit computations seem to indicate that this is the rule rather than the exception for planetary orbits. □

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New eyes for old

ALL of us, as we grow older, lose our ability to see closely in sharp focus. Bifocal, trifocal and even distributed-focal-length glasses are very imperfect solutions. Daedalus once invented a variable-focus pair of spectacles whose flexible plastic-film lenses could be pumped up with water to change the focal length — rather like the lens of the eye itself. He now returns to this idea with the crucial advance needed to make it practical and popular. His new variable-focus spectacles can focus themselves automatically.

After some thought, he has rejected the autofocus techniques used by modern cameras. His Autospecs do not measure the distance of the subject by means of an infrared beam; nor do they form an image and optimize its high spatial-frequency contrast. Instead, they take their cue from the eye itself. As the lens beneath the cornea focuses under the wearer's optical reflexes, its contour changes, and the cornea bulges or flattens in sympathy. Autospecs follow this process by means of small infrared sources on the spectacle frame, which bounce a pattern of beams off the surface of the eye to deduce its frontal curvature. The Autospecs then adjust their focus to aid the eye in its efforts. The whole arrangement acts as a fast-acting closed-loop servo system amplifying the wearer's own instinctive focusing. He will find his accommodation range magically extended. The proportion of focusing effort taken over by Autospecs will depend on their loop-gain. With sufficient loop-gain, they will restore focal power to almost fixed-focus eyes. As long as the wearer's instinctive effort to focus is perceptible at the cornea, the new glasses will sweep through their focal range until he is seeing sharply, and that effort relaxes.

This new technology will be widely welcomed. Bifocal glasses, which trade strained eyes for an arthritic neck, will rapidly vanish. Daedalus's prototype is rather heavy and clumsy, combining as it does corrective glass lenses, variable-focus liquid lenses, and quite a lot of electronics, but DREADCO's opticians are working hard to miniaturize it. They hope to give it a focal range far exceeding that of the eye itself, letting the wearer focus as closely and sharply as if he had a magnifying glass. Watchmakers, instrument mechanics, stamp-collectors and entomologists will be especially grateful. Daedalus even hopes for large sales among the young, who can focus quite well unaided. With his Autospecs focusing for him, the lenses of a youthful wearer will not be fatigued and work-hardened over the years by constant flexing. He will enter his middle years with eyes so wonderfully youthful that they may never lose their natural accommodation at all.

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1. Lighthill, J. *Proc. R. Soc. A* **407**, 35–50 (1986).
2. Laskar, J. *Nature* **338**, 237–238 (1989).
3. Nobili, A.M. in *The Few Body Problem* (ed. Valtanen, M.J.) 147–163 (Kluwer, Dordrecht, 1988).
4. Sussman, G.J. & Wisdom, J. *Science* **241**, 433–437 (1988).
5. Laskar, J. *Astron. Astrophys.* **198**, 341–362 (1988).
6. Poincaré, H. *Les Méthodes Nouvelles de la Mécanique Céleste* Vol. II (Gauthier-Villars, Paris, 1893).
7. Arnold, V. *Russian Math. Surv.* **18**, 85–191 (1963).

Distemper virus in Baikal seals

SIR—An acute disease of Lake Baikal seals (*Phoca sibirica*) attributable to morbillivirus infection became evident in the autumn of 1987 when weakened seals crawled onto the lake's icy shores and died. Many of them had paralysed hind extremities and ophthalmitis. No similar event is known from records going back to the 1930s. By October 1988, several thousand of the 80,000–100,000 seals in Lake Baikal had died.

In December 1987, during an expedition to Ushkani Island on the border between middle and northern Baikal, one of us (V.S.K.) noticed that three of the five dogs that lived there and had close contact with seals had died with typical symptoms of canine distemper virus (CDV) infection.

In the same month, we observed signs of a similar disease in a 6-year-old female seal (code PB-6). Two days after capture the animal seemed to have recovered but after a further 3 days it again showed symptoms of acute disease — diarrhoea, ophthalmitis and convulsions of the hind flippers. It died 10 days after capture. Rectal temperature varied between 34 °C on days 1 and 10, and 24 °C on days 5–7. No severe pathology of internal organs was observed but cytological investigations of bladder epithelium showed intracytoplasmic eosinophilic inclusion bodies characteristic of canine distemper¹. Inclusions were also found in cells of liver, kidneys, spleen, lungs and in neurons of the brain and spinal cord.

When the sera of eight dead seals were studied, six gave weakly positive indirect haemagglutination reactions (titres of 1:10 to 1:80) with measles antigen², which is a close relative of CDV; all sera neutralized the cytopathic effect of CDV vaccine strain EPM³ with titres from 1:8 to 1:64.

To prove the presence of a CDV-like virus in seal tissues, we have recently used the technique of oligonucleotide probing. On the basis of the known sequences of CDV RNA^{4,5} we synthesized 14–40

deoxyoligonucleotide probes complementary to either virion RNA, or to the corresponding messenger RNA. The probes were designed to detect sequences that are either common to CDV and measles virus (probes 1–7, for gene *N* sequences, and probes 14–19 for gene *P* sequences), or sequences that differ between the two viruses (probes 8–13 for the gene *N* sequences that differ most). The precise sequences probed are shown in *a* in the figure. Probing revealed both virion and mRNA sequences in seal PB-6. These data suggest that CDV or a close relative was not only present but biologically active, inducing synthesis of its mRNA. These results and the preliminary immunological data were discussed at a conference held in Irkutsk in February 1988.

In June 1988, several hundred samples of blood and tissues of Lake Baikal seals were obtained during the spring cull. Of 83 spleen samples subjected to oligonucleotide probing, 8% gave a strong positive signal for CDV RNA and there was a total of 55% positive samples. The 45% negative samples confirmed that the assay is selective (*b* in the figure).

Along with CDV-neutralization tests, we also performed both radioimmuno-metric assays, using CDV vaccine absorbed on nitrocellulose and rabbit anti-seal IgG labelled with ¹²⁵I, and enzyme-linked immunosorbent assays (ELISA), using a monolayer of Vero cells infected with CDV in plastic wells and protein A conjugated with peroxidase⁶. A large proportion of animals tested contained antibodies against CDV in their blood.

In the neutralization assay, 55% of the sera had titres between 1:16 and 1:64; 50% were positive in the radioimmuno-metric assay; and 89% were positive by ELISA, with titres from 1:16 to 1:1,280. These data indicated that the whole population of Lake Baikal seals had been in contact with the virus, suggesting that the survivors should be immune to further infections. The lack of any unusual mor-

ality of seals in the autumn or winter of 1988 confirms that immunity is now established.

We mentioned⁷ CDV as the cause of the Baikal seal disease in September 1988 when, first from newspapers, and then from Dr A. Osterhaus, we learned that CDV or a closely related virus was also believed to have caused the disease of seals in Western Europe. This has been confirmed by several techniques^{8,9}. According to nucleic acid hybridization and immunological data^{10,11} the virus in European seals is not identical to CDV but a distinct seal virus (phocid distemper virus; PDV). Our data on Baikal seals prove only that they were infected by a morbillivirus closely related to CDV; its precise relationship to other morbilliviruses will only be established when the seal virus RNA has been sequenced. The fact that seals in Baikal and in Western Europe have been infected by very similar, or identical, viruses poses new questions concerning the origin and spread of the seal virus.

During a very recent visit, Dr A. Osterhaus brought us sera of European seals, some of which contained antibodies (with titres up to 1:1,080) to CDV in our ELISA. These data and those reported below by Osterhaus *et al.* strongly suggest that the Baikal and European seals have been infected by the same virus, or one that is closely related.

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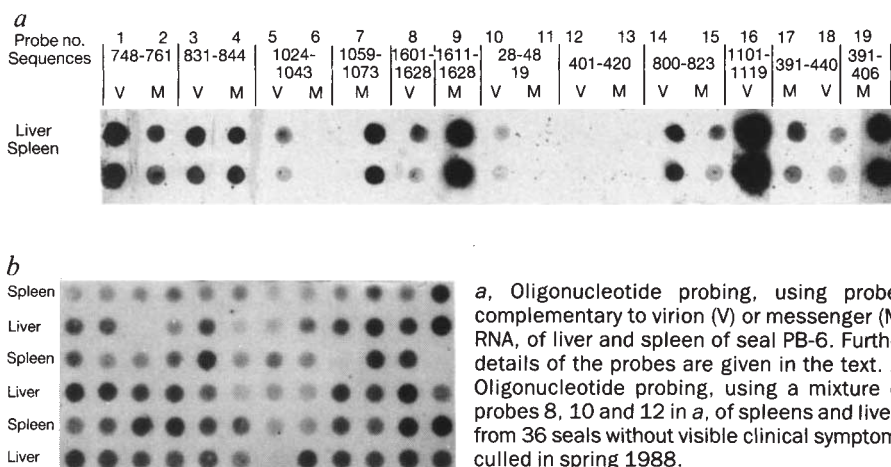
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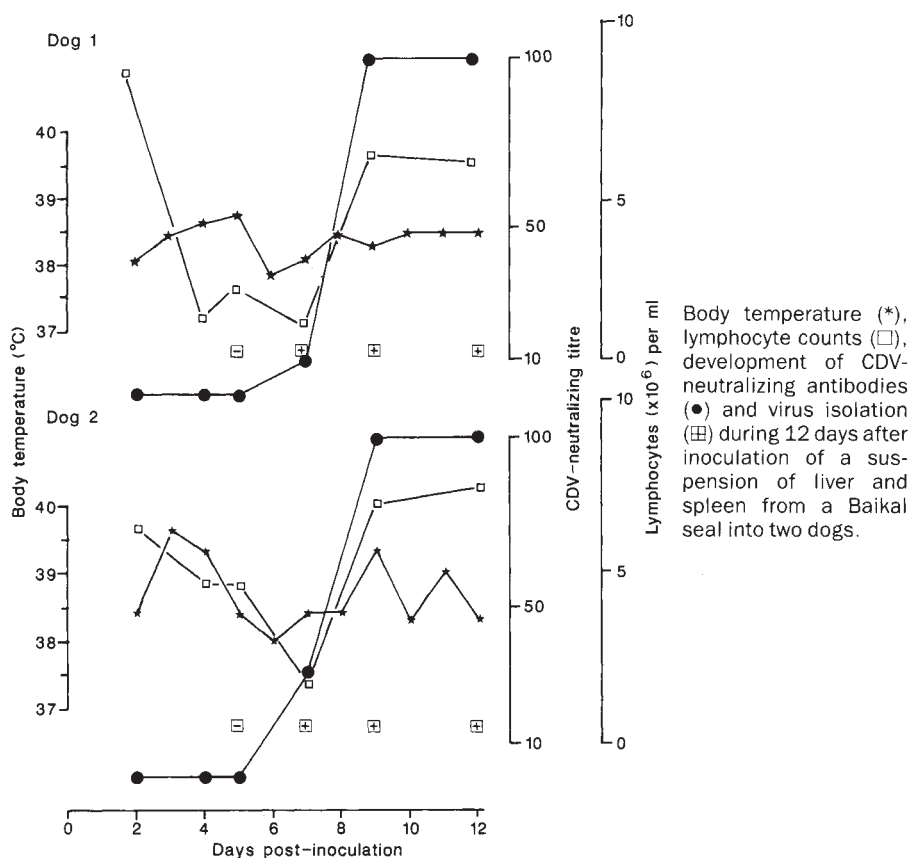
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SIR—We have demonstrated the presence of an infectious morbillivirus in the organs of a Baikal seal (*Phoca sibirica*) collected on a recent visit to Lake Baikal.

The seal was one of three that had suffered from a canine distemper-like disease,



a, Oligonucleotide probing, using probes complementary to virion (V) or messenger (M) RNA, of liver and spleen of seal PB-6. Further details of the probes are given in the text. *b*, Oligonucleotide probing, using a mixture of probes 8, 10 and 12 in *a*, of spleens and livers from 36 seals without visible clinical symptoms culled in spring 1988.



had the typical histopathological lesions of the disease in harbour seals (*P. vitulina*), and whose serum contained canine distemper virus (CDV)-neutralizing antibodies. Samples (2 ml) of a 10 per cent mixture of liver and spleen suspension were inoculated into two 12-week-old specific pathogen-free Beagle dogs, as previously described¹. The animals were observed for the development of clinical symptoms and tested for the development of CDV-neutralizing and ELISA antibodies in their sera, for 12 days. As shown in the figure, both dogs developed essentially the same mild clinical symptoms as dogs inoculated with material from the European harbour seals, including a slight body temperature rise and lymphopaenia within 1 week, and mild respiratory symptoms including mucopurulent nasal discharge within 2 weeks. Antibodies were detectable within 7 days, and the presence of a morbillivirus in peripheral blood mononuclear cells of both dogs on days 7, 9 and 12 post-inoculation was demonstrated as previously described in the above reference.

These data clearly confirm earlier speculations that a morbillivirus identical with, or similar to, the virus that struck north-west European harbour seals, had previously infected and caused canine distemper-like symptoms in Baikal seals. We hope to compare the biological and molecular properties of the morbilliviruses isolated from the Siberian and European outbreaks in an attempt to discover whether there was an epizootiological link

between the two outbreaks. If so, the morbillivirus may have spread from Siberia to Europe within 6 months either by terrestrial carnivores or by means of the extensive bird migration between Siberia and Europe.

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1. *Veterinarian Encyclopaedic Dictionary*, 572-573 (Soviet Encyclopedia, Moscow, 1981).
2. Izibicky, A. Z. *Immunoforsch.* **140**, 279-282 (1970).
3. Dorofeev, V. M. et al. FRG patent no. 2991040.
4. Rosenblatt, S. et al. *J. Virol.* **53**, 684-690 (1985).
5. Barrett, T. et al. *Virus Res.* **3**, 367-372 (1985).
6. Rice, G. P. A. et al. *J. infect. Dis.* **147**, 1055-1059 (1983).
7. *New Scientist*, No. 1631 (1988).
8. Osterhaus, A. D. M. E. *Nature* **335**, 20 (1988).
9. Osterhaus, A. D. M. E. et al. *Nature* **335**, 403-404 (1988).
10. Mahy, B. W. J., Barrett, T., Evans S., Anderson, T. C. & Bostock, C. J. *Nature* **336**, 115 (1988).
11. Cosby, S. L. et al. *Nature* **336**, 115-116 (1988).

Time asymmetry

SIR—Wolpert's reservations¹ on the 'Brussels School' approach to the problem of irreversibility, as summarized in Coveney's review², require a response.

Wolpert asks "why the underlying (time-symmetric) physics should imply the (overtly time-asymmetric) equations"? It is now well understood that it is precisely the instability of the underlying conservative reversible dynamics, which leads to the realization of the unitary group evolution, as a semigroup breaking the temporal symmetry.

As an example, let us consider the Kolmogorov systems, also mentioned in Coveney's review. These systems are characterized by a stable and an unstable partition of the initial data into cells evolving asymmetrically in time, under the conservative dynamical evolution. The remarkable fact about these partitions is that they are self-propagated by the dynamics, and not destroyed, as ordinary coarse graining partitions would be. The cells of the stable or K partition become refined in the future and coarser in the past, whereas the cells of the unstable partition experience the reverse behaviour under the dynamical evolution.

Therefore, for Kolmogorov systems an arrow of time is intrinsically built in, by observing the time-asymmetric evolution of the partitions. For these systems the second law is expressed as a selection principle of initial conditions. Only initial data leading to equilibrium in the future may be realized. The selection principle of the future arrow corresponds to a non-unitary realization of dynamics as a Markov process. This realization results from the operational limitations due to the continuous refinement under the dynamical evolution, to controlling or distinguishing trajectories in the same cell of the stable K partition. For these reasons K systems have been called intrinsically irreversible^{3,4}. Therefore, Wolpert's first requirement — that the underlying dynamics be reversible — is automatically satisfied for the conservative K systems.

Concerning Wolpert's second requirement "why nature in fact chooses one direction in time over the other", for K systems there exist two semigroups, one leading to equilibrium in the future and the other in the past. The choice of the semigroup leading to equilibrium in our future corresponds to our physical experience. It is the basis of the formulation of the second law of thermodynamics: entropy increases in the direction of our future. The existence of a unique privileged direction of time for all processes in our observable universe refers to the compatibility of the future arrow of intrinsically irreversible dynamical systems with the cosmological arrow of time. (For a recent discussion of the cosmological

problem see ref 5.)

Wolpert returns to old criticisms of Boltzmann's pioneering work. In view of the recent developments of unstable dynamical systems it is now well understood⁶ that the objections raised against Boltzmann, such as the Zermelo paradox, are not meaningful, as the Poincaré recurrence time is much larger than the Liapunov time, being the time after which we lose control over the trajectories of a dynamical system.

In short, recent developments, including those of the Brussels School, permit one to incorporate the second law into the formulation of dynamics for highly unstable dynamical systems. In this way, the seeming contradiction between the reversibility of dynamics and the irreversibility of the second law has been overcome.

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1. Wolpert, D. *Nature* **335**, 595 (1988).
2. Coveney, P. *Nature* **333**, 409 (1988).
3. Prigogine, I. *From Being to Becoming* (Freeman, San Francisco, 1980).
4. Misra, B. & Prigogine, I. in *Long Time Predictions in Dynamical Systems* (eds Horton, C., Reichl, L. & Szebehely, V.) 21–43 (Wiley, New York, 1983).
5. Prigogine, I., Gheniau, J., Gunzig, E. & Nardone, P. *Proc. natn. Acad. Sci. U.S.A.* **85**, 7428–7432 (1988).
6. Lighthill, J. *Proc. R. Soc. A* **407**, 35–50 (1986).

'Amylin' hormone

SIR—Leighton and Cooper¹ recently reported a potential physiological function of a newly discovered pancreatic β -cell hormone which they refer to as 'amylin'. The introduction of the term amylin is a source of potential confusion because this peptide has also been called islet amyloid polypeptide (IAPP)^{2,4} and diabetes-associated peptide (DAP)⁵. This fact prompts us also to point out recent progress in relating IAPP/DAP/amylin, which we prefer to call IAPP, to type-2 diabetes (age-related diabetes mellitus).

The 37-amino-acid IAPP is the main component of amyloid deposits of the islets of Langerhans occurring in association with type-2 diabetes in humans and cats^{2,5}. It is a normal product of pancreatic β -cells^{3,6,7} that is colocalized and probably cosecreted with insulin^{8,9} and generated through proteolytic processing of an 89-amino-acid precursor (ref. 10; C. Betsholtz *et al.*, unpublished data).

IAPP inhibits insulin-stimulated glycogen synthesis in skeletal muscle preparations *in vitro*¹. This is interesting in relation to the pathogenesis of type-2 diabetes because this disease is characterized by peripheral insulin resistance in addition to islet dysfunction.

That amyloid formation is of importance to the pathogenesis of type-2 diabetes is implied by the correlation

between the presence of an amino-acid motif in the IAPP molecule and both amyloid formation and the spontaneous onset of type-2 diabetes in adult humans, cats and racoons; this motif is lacking in species that do not develop islet amyloid or type-2 diabetes (C. Betsholtz *et al.*, unpublished data).

Thus, changes in IAPP synthesis, processing or release may relate to both major traits of the type-2 diabetic syndrome; islet dysfunction through amyloid formation and insulin resistance through a peripheral action.

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1. Leighton, B. & Cooper, G.J.S. *Nature* **335**, 632–635 (1988).
2. Westermark, P., Wernstedt, C., Wilander, E. & Sletten, K. *Biochem. biophys. Res. Commun.* **140**, 827–831 (1986).
3. Westermark, P. *et al. Proc. natn. Acad. Sci. U.S.A.* **84**, 3881–3885 (1987).
4. Westermark, P., Wernstedt, C., O'Brien, T.D., Hayden, D.W. & Johnson, K.H. *Am. J. Path.* **127**, 414–417 (1987).
5. Cooper, G.J.S. *et al. Proc. natn. Acad. Sci. U.S.A.* **84**, 8628–8632 (1987).
6. Westermark, P., Wilander, E., Westermark, G.T. & Johnson, K.H. *Diabetologia* **30**, 887–892 (1987).
7. Cooper, G.J.S. *et al. Lancet* **ii** 966 (1987).
8. Johnson, K.H. *et al. Am. J. Path.* **130**, 1–8 (1988).
9. Lukinius, A., Wilander, E., Westermark, G.T., Engström, U. & Westermark, P. *Diabetologia* (in the press).
10. Sanke, T., Bell, G.I., Sample, C., Rubenstein, A.H. & Steiner, D.F. *J. biol. Chem.* **263**, 17243–17246 (1988).

On the SCIDs?

SIR—We recently reported (*Nature* **335**, 256–259; 1988) that a functional human immune system could be established effectively in mice with severe combined immunodeficiency (SCID mice) by inoculating them intraperitoneally with peripheral blood leukocytes (PBL) of human origin. The major immunological conclusions of that study are, first that recipient mice show prolonged survival with no ill effects of graft-versus-host disease; second, that the human leukocytes, including both B and T lymphocytes, increase in number and survive in these mice now for at least one year; third, that the reconstituted mice show significant levels of spontaneously secreted human immunoglobulin; and, last, that specific human antibody responses are inducible by immunization. These have all been confirmed in subsequent studies involving many more PBL-reconstituted mice.

More recent studies, however, have indicated that the numbers of intraperitoneally inoculated human PBL that migrate to the spleen, lymph nodes and

peripheral blood during the first five-week period after reconstitution may be significantly less than those reported in Fig. 3 of our paper. For reasons not yet fully understood, antibodies used for detection of human cells show high levels of non-specific binding to splenic cells in these mice soon after reconstitution. Nevertheless, human cells do appear in these lymphoid organs in larger numbers at later times.

We caution other workers to be alert for this staining artefact, and we regret any inconvenience that our overestimates may have caused.

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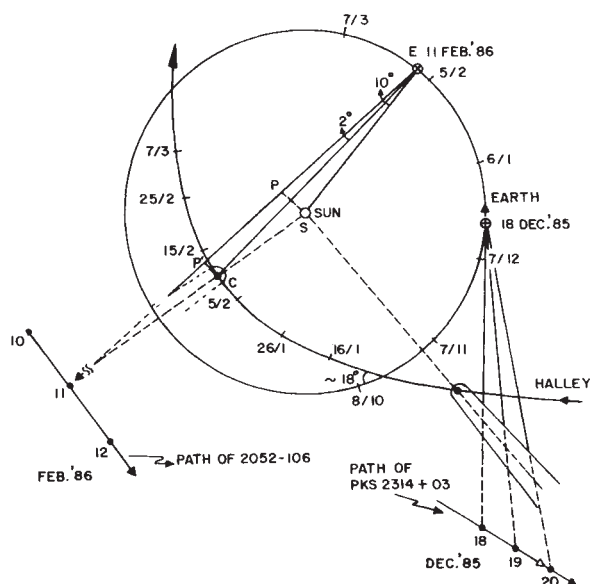
Quasar enhanced

SIR—During the recent appearance of Halley's comet, observations were reported^{1,2} of enhanced scintillations of quasars occulted by the cometary ion-tail; however, Ananthakrishnan *et al.*³ dispute such an origin for these scintillations.

The geometry at the time of both the 103-MHz observations¹ on 18–20 December 1985, and those at 327 MHz (ref. 3) on 10–12 February 1986, is depicted in the figure. On 18 December 1985, the source, PKS2314+03, lay at a solar elongation of about 85°, such that the solar wind contributed only to background scintillations, with an average scintillating flux of about 3 Jy (ref. 1). The observed enhancement was six times this value and 1.5 times the maximum flux normally caused by the solar wind, which, for 103 MHz, occurs at a solar elongation of about 30°.

During the 327-MHz observations³ on 11 February 1986, the source 2052–106 was presumed to be occulted by the cometary tail. As the perihelion occurred on 9 February, the period 10–12 February is most inappropriate for observing such events: the solar elongation of 2052–106 on 11 February was about 10°, for which, at 327 MHz, strong scattering prevails. The scintillation index is near its maximum at around 14°. Consequently, most of the scattering occurs in a thin layer of the solar plasma centred around the point of closest approach to the line of sight from the Sun. The three control sources were also within 12° of the Sun. Therefore, the scintillation enhancements of all of the sources were due solely to the solar plasma. The source also gets broadened by strong scattering close to the Sun and enhanced outflow of cometary plasma, which results in reduced scintillation. These conditions are therefore unsuited to observing enhancement of scintillations by Halley's comet.

Thus, enhanced scintillations of



Relative positions of the Sun, the Earth and Halley's comet during the observations reported in refs 1 (December 1985) and 3 (February 1986). The paths of the quasar sources are also shown.

compact radio sources resulting from occultation by strong cometary ion-tails can be identified only for favourable alignments of the comet, Sun and observer. Such conditions existed during the observations reported in ref. 1 and it is possible, therefore, that the scintillations were of cometary origin. Unambiguous confirmation could be obtained by using more control sources close to the line of sight⁴.

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ANANTHAKRISHNAN *ET AL.* REPLY—The importance of our letter¹ lies in its demonstration that control sources are essential in investigations of scintillation through the cometary plasma tail. Alurkar *et al.* also agree that control observations are essential. Without such observations it is difficult to determine whether or not the observed¹ enhancement in scintillation is caused by the plasma tail.

We disagree with the suggestion that because our observations of 2052-106 on 11 February 1986 were made at a solar elongation of about 10° (actually 11.2°), the circumstances were unsuitable for studying the enhancement of turbulence. On the contrary, the outflow of plasma from the comet would be an order of magnitude higher for our observations than for those of Alurkar *et al.*¹, made at a solar elongation of 85° and before perihelion. Furthermore, angular broadening effects are unimportant unless the source is very much closer to the Sun. Therefore, the 60% enhancement seen by us (Fig. 2 of ref. 1) could have arisen either from the comet or from the solar wind. However, a similar increase in the control source implies that a solar-wind origin is most probable. In addition, observations far from the Sun, of the sources 1817-391

and 1921-293 along with control sources, also show no enhancement resulting from the plasma tail³.

We agree that geometry of occultation is important. Unfortunately, cometary occultations of the required geometry are extremely rare, and consequently there is a need to take every available future opportunity for making occultation observations of radio sources by cometary tails (along with suitable control sources) in order to resolve the present conflict of results.

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1. Alurkar, S.K., Bhonsle, R.V. & Sharma, A.K. *Nature* **322**, 439-441 (1986).
2. Slee, O.B., McConnel, D., Lim, J. & Bobra, A.D. *Nature* **325**, 699-701 (1987).
3. Ananthakrishnan, S., Manoharan, P.K. & Venugopal, V.R. *Nature* **329**, 698-700 (1987).
4. Hajivassiliou, C.A. & Duffett-Smith, P.J. *Mon. Not. R. astr. Soc.* **229**, 485-493 (1987).

Zinc and LTP

SIR—Recent studies have implicated *N*-methyl-D-aspartate (NMDA) receptor activation and concurrent postsynaptic depolarization in the induction of long-term potentiation (LTP)¹. Kauer *et al.*² recently showed that NMDA receptor activation produced only a transient potentiation in the CA1 region of the hippocampus and speculated that some additional factor, perhaps co-released with glutamate from presynaptic terminals, was essential for the later, long-lasting phase of LTP. We suggest that synaptic Zn²⁺ is the factor. Zn²⁺ is systematically localized in synaptic vesicles of excitatory synapses throughout the mammalian telencephalon³ and probably is co-released with glutamate into the synaptic cleft with neuronal activity^{4,5},

especially at higher rates of stimulation⁶. Zn²⁺ decreases NMDA receptor-mediated responses but increases quisqualate receptor-mediated responses^{7,8}, and may acutely modulate the postsynaptic response to glutamate. If this synaptically released Zn²⁺ can gain entry into postsynaptic neurons, it is possible also that it could induce LTP. It has been shown *in vitro*, that Zn²⁺ can activate protein kinase C and induce its translocation to membranes⁹, both associated with establishing LTP^{1,2}. Entry of Zn²⁺ into neurons has not been clearly documented but it could pass through the NMDA channels. Zn²⁺ can block the NMDA channel (C. W. Christine and D. W. Choi, manuscript in preparation). Mg²⁺, and perhaps divalent cations in general, may permeate the NMDA channel at a rate determined by the ease with which surrounding water molecules can be lost^{10,11}. Zinc can shed its water molecules at a rate between that of Ca²⁺ and magnesium¹², suggesting that its permeability may be greater than that of Mg²⁺. The neurotoxicity of Zn²⁺ on cortical neurons is consistent with Zn²⁺ entry through the NMDA channel, as it is competitively blocked by the NMDA-channel blocker, MK-801, and increased by removal of extracellular Na⁺ and calcium¹³.

Thus the simple co-occurrence of NMDA receptor activation with postsynaptic depolarization could result in a calcium influx and transient postsynaptic potentiation. If the presynaptic neuron is firing sufficiently rapidly, Zn²⁺ could be released from presynaptic terminals and enter the postsynaptic neuron via NMDA channels, activating protein kinase C (and perhaps other intracellular processes) resulting in LTP.

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1. Brown, T. H., Chapman, P. F., Kairiss, E. W. & Keenan, C. L. *Science* **242**, 724-728 (1988).
2. Kauer, J. A., Malenka, R. C. & Nicoll, R. A. *Nature* **334**, 250-252 (1988).
3. Perez-Clausell, J. & Danscher, G. *Brain Res.* **337**, 91-98 (1985).
4. Assaf, S. Y. & Chung, S. H. *Nature* **308**, 734-736 (1984).
5. Howell, G. A., Welch, M. G. & Frederickson, C. J. *Nature* **308**, 736-738 (1984).
6. Aniksztejn, L., Charton, G. & Ben-Ari, Y. *Brain Res.* **404**, 58-64 (1987).
7. Peters, S., Koh, J. & Choi, D. W. *Science* **236**, 589-593 (1987).
8. Westbrook, G. L. & Mayer, M. L. *Nature* **328**, 640-643 (1987).
9. Cserehely, P., Szamel, M., Resch, K. & Somogyi, J. J. *biol. Chem.* **263**, 6487-6490 (1988).
10. Ascher, P. & Nowak, L. J. *Physiol. Lond.* **399**, 247-266 (1988).
11. Mayer, M. L. & Westbrook, G. L. *Soc. Neurosci. Abstr.* **11**, 785 (1985).
12. Diebler, H., Eigen, M., Ilgenfritz, G., Maas, G. & Winkler, R. *Pure appl. Chem.* **20**, 93-115 (1969).
13. Koh, J. & Choi, D. W. *Soc. Neurosci. Abstr.* **14**, 417 (1988).

The drug-reaction drama

Louis Lasagna

Responsibility for Drug-Induced Injury. By M.N.G. Dukes and D. Swartz. Elsevier: 1988. Pp.431. Dfl.350, \$184.25.

RARELY does a new book have no competitor; Dukes and Swartz have written such a book. The authors' backgrounds are complementary, combining science (including political science) and the law. Although the literature on drug-related injury is abundant, as is that on the litigious implications thereof, Dukes and Swartz concentrate here on the *responsibility* for drug-induced injury. The volume should enjoy brisk sales, at least among lawyers and employees of pharmaceutical companies.

As the opening paragraph of the introduction (after a somewhat pompous first sentence) points out, it is important to determine when and how a drug injury is preventable, and in this assessment we need "to find out what went wrong". Unfortunately, that is often much more easily said than done. The 'Elixir Sulfanilamide' story is the exception; the deaths it caused were easy to explain and could all have been prevented, had the toxic solvent only been tested in animals for safety. Consider, in contrast, the Japanese 'S.M.O.N.' story, a devastating epidemic of neurological disease, blamed on clioquinol, a drug sold widely all over the world without manifesting toxicity like that seen in Japan. Even in retrospect, clioquinol seems likely to have been at best a 'co-villain', perhaps facilitating damage to the central nervous system by some neurotropic virus. And what of thalidomide, with which 'seal babies' could be reproduced in animals only with great difficulty after the fact, with many species, breeds and strains of rabbits, mice, rats, hamsters, dogs and primates failing to show the dreaded anomalies?

Dukes and Swartz say that "several thousand asthmatic patients may have been killed by high dose isoprenaline aerosols". But these high-dose preparations have disappeared, and 'epidemics' of asthma deaths have occurred periodically since then in different countries — only now they are generally attributed to undertreatment (for example with corticosteroids), not drug toxicity. Even with drugs taken off the market (benoxaprofen and Indosmos, for instance), the numbers cited for deaths attributable to them are soft indeed; sick, elderly patients who die while on a drug are not necessarily victims

of the medication.

In an important introductory paragraph (p.6), the authors point out that in some countries (the United States being a prime example), there has been a tendency to seek compensation for injury from "the wealthiest or best-insured party (often the pharmaceutical industry) even when this has entailed redefining such considerations as foreseeability or retrospectively postulating the existence of a duty not



previously recognized". Bendectin (Debendox) was the only prescription drug ever approved in the United States for treating the nausea and vomiting of pregnancy. Lawsuits alleging that it was a teratogen forced its withdrawal from the marketplace worldwide, even though there have been repeated verdicts of 'innocent' from expert panels and courts. One can safely wager that no sponsor will ever again try to market a drug for this indication in the United States (even though an early Australian critic of the drug has admitted faking his data). Fears of litigation have in effect driven most large US companies out of vaccine research and development, despite the clear need for new vaccines both for civilian and military populations.

Dukes has criticized 'me-too' drugs that "were merely slight variants on existing molecules . . . which provided no new benefit but merely introduced new risk". But it must be a rare pharmaceutical company that purposely markets inferior variants in today's very competitive market. Furthermore, half of the World Health Organization's *List of Essential*

Drugs is made up of compounds that were not the first drugs in their therapeutic categories; many of these could be dubbed 'minor chemical modifications' of the breakthrough products. Would we really want to be restricted today to the earliest penicillins or the first sulphonamides?

The book accurately describes the complex basic problems in establishing causality, but does not adequately communicate the fact that certainty about the cause of a drug reaction (or even that one has occurred) is uncommon. Experts shown the data about a suspected reaction quite typically disagree vigorously — not least because multiple medications are often being taken by patients, and it is often impossible to rule out spontaneous comorbidity unrelated to drugs, or the possibility that the patient is manifesting another aspect of his basic illness. One cannot usually state categorically that an ingested drug could *not* have caused a given adverse effect, but it is only rarely possible to state that a given drug was unquestionably the cause. Algorithms to identify causality (an area which my colleagues and I pioneered) are useful primarily as check lists, not as ways to eliminate uncertainty.

This book is not easy reading, and there are variations in the size of type that are difficult to interpret (is small for less-important material?). But potentially 'responsible' parties will ignore it at their peril. The authors address the obligations of a long list of players in the drug-reaction drama: physicians, dentists, medical students, manufacturers, drug retailers, dispensing pharmacists, nurses, patients, governments and official agencies. (One wishes that the legal profession were sometimes more 'responsible'; trivial and capricious suits are by no means uncommon — at least in the United States.)

Dukes and Swartz dedicate their opus to the World Health Organization. One can question, however, their assertion that "materials emanating from the WHO are probably the most authoritative of all documents on drug issues, representing a broad consensus". In my own experience with WHO committees, I have been struck by the compromises made necessary by the fact that the representatives from different countries must hammer out a document with which they can live on their return home. Consensus based on politics is not necessarily the ultimate in scientific authority. □

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Ways of being catty

Juliet Clutton-Brock

The Domestic Cat: The Biology of Its Behaviour. Edited by Dennis C. Turner and Patrick Bateson. *Cambridge University Press*:1989. Pp.222. Hbk £35, \$59.50; pbk £15, \$22.95.

It is probably because domestic mammals have been held in a certain disdain by zoologists that studies of their behaviour have been so neglected. To observe the behavioural patterns of the leopard, in the wild, will enhance status; it requires large sums of money, complicated equipment and personal courage; to watch the behaviour of the domestic cat requires only time, patience and experience in interpretation, yet the results may yield a greater knowledge of felid behaviour. Perhaps more importantly, they will yield information on the relationships between human beings and their animal companions, a subject that has been submerged for too long in pejorative terms such as 'anthropomorphism' and 'pet-keeping'.

This symposium volume, edited by Turner and Bateson, is the first work to bring together much of the research that has been carried out, in recent years, on the biological basis of behaviour in the domestic cat. It includes reports of studies on reproduction, social life, predatory behaviour, feral cats and house cats, all carried out with exemplary attention to the codes of ethology.

One aspect of research on the cat that is missing from the book is the connection between the patination and colour of the coat and temperament (as distinct from the link between breed and temperament which is discussed by Mendl and Harcourt, p.48). Karsh (p.173), when asking what influenced people's choice of a cat, was told by one woman that the personality of her cat was all-important to her. However, she later claimed not to be able to relate to a cat that was all white or all

black, and that she related better to a cat that was grey or striped. Perhaps experience enabled her to predict what the general temperament of the animal would be from its pelage. In his article "Cats and Commerce" (*Scientific American* 237, 100-107; 1977), Todd has postulated that black cats may be less aggressive and more tolerant of crowding than striped tabbies, which might therefore have a more distinctive temperament. Clearly, there is a need for more work on this topic.

An underlying thread in the book is the need to quantify the characteristics of individuality, and perhaps the greatest contribution to ethology that can be made

from domestic animals will come from studies of behavioural variation. For unless a population of wild animals has been habituated, it is very difficult to categorize individual responses. People who live with cats know them as individuals; Mendl and Harcourt (pp.41-54) have made a start to defining individual variation and the biological significance of behavioural style.

The Domestic Cat is sure to become a classic in the study of behaviour in domestic animals. □

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Out in the open

J.R.S. Fincham

The Release of Genetically-Engineered Micro-Organisms. Edited by M. Sussman, C.H. Collins, F.A. Skinner and D.E. Stewart-Tull. *Academic*:1988. Pp.306. £29.50, \$59.50.

THE molecular cloning of genes and their transfer between different kinds of cells and organisms is now a standard analytical method, practised in almost every modern university biological department, and filling more than half of the pages of the leading biological journals. Anxiety about its safety was quite intense in some quarters in the late 1970s, but has largely died away.

Now we are faced with another wave of concern, this time arising from a wide range of schemes for putting genetically manipulated organisms to use outside the laboratory. Typically, this issue has generated less heat in Britain than in either the United States or continental Europe. This may, perhaps, have been a factor in the choice of Cardiff as the venue for the symposium from which this book stems. Sponsored jointly by the International Union of Microbiological Societies and three of its British and American constituents, it drew together contributions from a selection of the relevant university and institute-based scientists; scientists working in commercial laboratories were less well represented. The published proceedings include 13 papers, and about 100 pages are devoted to rapporteurs' accounts of eight round-table discussions.

The volume was hurried into print a mere seven months or so after the conference, and it suffers from most of the defects that make symposium proceedings (with a few distinguished exceptions) the most ephemeral and least worthwhile of library purchases. It lacks both the coherence of style and argument of a good book and the rigorous presentation of primary data that one expects in a good

journal. The articles are uneven in style, ranging from the unduly terse to the prolix. The round-table reports contain some of the most interesting information, but it tends to be reduced to the status of anecdote by the lack of references. The index is very inadequate.

Nonetheless, this particular symposium volume is likely to attract much interest as a source of up-to-date information about the kinds of environmental release that are in prospect and the purposes that they are intended to serve. The symposium was mainly concerned with three areas of application: agriculture (improved nitrogen fixation and better pest control); biodegradation of chemical wastes; and the development of safe live vaccines. There is only one article (by J.E. Davies) on the present and impending applications to chemical and pharmaceutical industry. Important as these undoubtedly are, they do not involve environmental release but rather scaled-up indoor containment.

A theme running through several of the contributions is the need to ensure that any introduced micro-organism does not persist indefinitely in the environment. Roy Curtiss III proposes various non-lethal mutations, such as deficiency in cyclic AMP, which may militate effectively against long-term survival of engineered bacteria. D.H.L. Bishop *et al.*, in tests of a baculovirus intended for use as a narrow host-range insecticide against a moth, found that a mutant unable to make the polyhedral viral coat was still infectious but did not persist in the soil or on plant surfaces. It is intended, by further engineering, to make the virus even more deleterious to the moth than it is already, but this aspect is not dealt with in the book.

The main point of the article by S.E. Lindow and N.J. Panopoulos is that the now famous ice-minus *Pseudomonas syringae* does not survive well in the soil. Studies on persistence of released bacteria depend, of course, on efficient methods for detection and monitoring, the theme of the article by R.R. Colwell *et al.* Enumeration of soil bacteria by colony

New in paperback

- *Infinite in All Directions* by Freeman Dyson (Bessie/Harper & Row, New York). Price is \$8.95. For review see *Nature* 332, 748 (1988).
- *On Human Nature* by Edward O. Wilson (Harvard University Press). Price is \$8.95, £7.25. For review see *Nature* 276, 120 (1978).
- *Franklin of Philadelphia* (a biography of Benjamin Franklin) (Harvard University Press). Price is \$12.95, £10.50. For review see *Nature* 322, 23 (1986).
- *Schrödinger: Centenary Celebration of a Polymath* edited by C. W. Kilmister (Cambridge University Press). Price is £12.50, \$22.95. For review see *Nature* 326, 913 (1987).

counts depends on having specific selectable markers. Antibiotic resistance genes are generally unacceptable for environmental release but the *E. coli lacZ/Y* genes, which lend themselves to easy visual detection, are shown by D.J. Drahos *et al.* to provide a useful alternative. The most sensitive method of detection is probably to probe for a DNA sequence specific to the target organism after amplification of that sequence in the sample under test by the polymerase chain reaction. This does not, however, give a quantitative measure of abundance prior to amplification.

The promise of human benefit emerges most clearly in the articles on the development of new vaccines. A.A. Lindberg deals with vaccines against oral and enteric pathogenic bacteria, and E. Paoletti and his associates with the use of fowlpox virus vectors to vaccinate non-avian species. In one of the round-table discussions, E. Wedman reports vaccination of calves against Sinbis virus (not, in fact, a pathogen) with a recombinant between Sinbis and vaccinia.

There is nothing on the use of vaccinia virus as a vector for the expression of genes encoding immunogenic proteins of pathogens, except for a full documentation of the unhappy affair of the rabies vaccine trial, carried out on cattle in Argentina. The perfectly respectable sponsors of the trial had failed to obtain clearance from the local authorities, and incurred universal denunciation. Clearly, innovators in this area need to have both a proper regard for safety and acute political sensitivity. Live vaccines produced by genetic engineering, though likely to be safer than the old vaccines obtained by attenuation of pathogenic viruses and brought into use on a trial-and-error basis, will doubtless have to satisfy far more stringent safety criteria before being accepted.

Those seeking controversy will be disappointed in this volume. Although there is preoccupation with containment, nobody puts forward any scenario of disaster should containment fail. There are a number of rather coy references to 'perceived' risks, but the perceivers do not appear to have been present at the symposium. Although many of the public expressions of concern have undoubtedly been irrational or perhaps (as in the furore about the ice-minus bacteria) merely symbolic, it is difficult to take efficient precautions if one does not have some idea of what one is guarding against. No doubt the scientists present did have their own perceptions of hazard, or lack of it. It would have made for a better-balanced symposium had these been more explicitly discussed. □

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Notes from the quantum field

Kate Metropolis & Chris Quigg

Beamtimes and Lifetimes: The World of High Energy Physicists. By Sharon Traweek. *Harvard University Press: 1988.* Pp.187. \$20, £15.95.

IF A coprophagous coleopteran stopped to reflect, its life would no doubt seem a noble quest. Nevertheless, the attention lavished upon the dung beetle by a *Serious Entomologist* would be flattering in the extreme, applauding — by reason of the entomologist's devoted interest — this scarab's life work. So it is with high-energy physicists and anthropologists: the life of the mind, the quest for nature's secrets, is suffused with its own divinity. But surely, to be taken seriously by serious persons must be the sincerest form of flattery. Sharon Traweek is a *Serious Anthropologist* and our colleagues must needs be flattered by her fascination with their habits and tribal customs.

The community Professor Traweek has chosen to study, that of experimental high-energy physicists, seems apt for classical anthropological analysis. The group has a shared past, anticipates a shared future, has rites for initiating new members and is able to distinguish itself from other communities. The population is divided into highly ordered, long-lasting associations: the handful of high-energy accelerator laboratories comprise several groups, each executing an experiment in its own style. A group consists of 50 to a few hundred physicists from different institutions, with varying backgrounds, specialities and levels of experience — graduate students, postdoctoral fellows, professors, laboratory staff members — who build a detector to gather data on subatomic particles and then analyse the information to construct a more complete picture of the Universe. Subgroups, sometimes individuals, are responsible for different parts of the hardware, software and analysis. Although people occasionally move between experiments and laboratories, the collaboration may last for a decade or more. The society is highly ordered, rich in mythology and customs; and, because there are communities of experimental high-energy physicists around the world, it offers anthropologists the opportunity to distinguish behaviour patterns endemic in the profession from expressions of national or ethnic traits.

Particle physics has well-known personalities — clowns, tyrants, thoughtful moralists, egomaniacs, wizards — about whom many people are curious. Are they typical? How do they interact? What order underlies a group in which the

list of workers on a single experiment may fill an entire page of a journal? An analysis that revealed the strengths and weaknesses of a particular approach to large collaborations could be interesting, amusing and even cautionary for scientists in fields beginning to collectivize. Indeed, Traweek comes upon provocative questions: What are the norms of behaviour within the community? How are actions and motives analysed? Why do people who see each other day in, day out recount different versions of history?

Scientific inquiry requires that researchers play Devil's Advocate until they can convince themselves that their data are complete and can be trusted. Unlike the physicists she studies, Traweek evinces no scepticism towards her data and fails to convince us that she has gathered anything more than an idiosyncratic sample. *Beamtimes and Lifetimes* reads less like a mature analysis than like a collection of undigested field notes recording observations at SLAC, the Stanford Linear Accelerator Center in the United States, and KEK, the national high-energy physics laboratory in Japan.

Traweek consistently uses the experience or opinions of a few individuals to draw broad conclusions about the entire community. If these illustrations were used to vitalize an observation or conclusion, they would be useful. Without a convincing richness of supporting data, however, her inductions have a foundation of quicksand. Further, her decision not to identify her subjects means that they become interchangeable: one group leader, one laboratory director is presented as indistinguishable from another. Yet in fact these people have quite different personal and scientific styles. She does not make a plausible case for not differentiating them, so the reader can only conclude that her powers of observation are not equal to her task.

This judgement is reinforced by Traweek's methodology. Reporting a discussion of how wives of high-energy physicists supported their husbands' work, she writes, "The older wife of a laboratory director interjected that she thought the way to avoid distracting the husband from his important work was to pursue one's own interests seriously. By their glances at each other I gathered that the younger women disagreed". A moment's reflection provides a number of alternative interpretations: interest in each other's reaction to an unfamiliar point of view, exasperation over hearing this position put forth for the millionth time by someone in a situation different from theirs, and so forth.

A recurring theme for Traweek is gender bias and sexism in the education, the rites of passage and the practice of experimental high-energy physics — a subject that should not be trivialized. If

particle physics *is* a male culture (although saying so doesn't make it so), it is important to understand how it came to be that way and why it remains true. Traweek's summation is as cogent as the evidence that Beatles records, played backwards, contain messages of devil worship:

The language used by physicists about and around detectors is genital: the imagery of the names SPEAR, SLAC, and PEP is clear, as is the reference to the "beam" as "up" or "down." One must see the magnets at LASS to appreciate the labial associations in the detector's name, Large Aperture Solenoid Spectrometer. Ironically, the denial of human agency in the construction of science coexists with the imaging of scientists as male and nature as female. Detectors are the site of their coupling: standing on the massive, throbbing body of the eighty-two-inch bubble chamber at SLAC while watching the accelerated particles from the beam collide twice a second with superheated hydrogen molecules made this clear to me.

The plethora of facts that Traweek has got wrong does nothing to encourage faith in her inductions. Descriptions of scientific apparatus, events and phenomena, intended to confer verisimilitude, are

grossly inaccurate. Her judgements are no more convincing: the statement that the food at SLAC is "rather good, for a cafeteria" will come as a shock to anyone who has eaten there.

The neglect of the reasons that inspire people to join and remain in this community is a crippling omission. Particle physics is not a closed or isolated society in which membership is determined by happenstance. Because the only motive Traweek recognizes is career advancement, her narrative is oblivious to the excitement, the exhilaration of doing physics. Nowhere do we see people driven by curiosity, intoxicated with the creation and understanding of new experience.

Beamtimes and Lifetimes is not a complete or reliable guide to the community of particle physicists. Let it be taken nevertheless as an invitation to particle physicists to re-examine their values, institutions and assumptions about each other and about the rest of the world. □

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Life transformed

Adrian Desmond

The Age of Lamarck: Evolutionary Theories in France 1790–1830. By Pietro Corsi. Translated by Jonathan Mandelbaum. University of California Press: 1988. Pp. 360. \$42.

AT THE entrance to the Jardin des Plantes, opposite the Seine, stands an imposing statue of Lamarck, the "Founder of the Doctrine of Evolution". And in relief, at the back, is a touching scene depicting a lonely, dejected Lamarck being consoled by his sister. This image, of an isolated trailblazing figure, is surely one of the most enduring in the history of biology.

Now Pietro Corsi, in his stunning study of Parisian science after the Revolution, shatters this myth. He questions the long-standing assumption that Lamarck was a prophetic genius, striding alone, out of time and place. The truth was very different; so much so that Corsi actually talks of an 'evolutionary culture', of which Lamarck was but a part. And in investigating this culture, he has given us the most compelling account yet of French transformatism between 1800 and 1830.

Historians have been quick to deny Lamarck an audience. But Corsi argues that the eighteenth-century buffonian tradition — with its grand, unifying view of nature and attempt to look at life through time — still had a populist appeal, even after the Revolution. And it was to the buffonians (most by then outside acad-

emia) that Lamarck spoke — to the marginal men appalled by the increasing specialization of science. They deplored Cuvier's attempt to subordinate natural history to a descriptive, analytical comparative anatomy. There were still plenty of these outsiders in 1800, criticizing Cuvier for failing to investigate the larger laws of nature. It was against this cultural background that Lamarck's own ambitiously theoretical works, with their rival approaches to *biologie* (his word), were formulated.

Lamarck started out as a botanist, chemist and conchologist, and he was appointed the professor of insects and worms at the Muséum d'Histoire Naturelle in 1793. Before 1800 he remained a staunch anti-transformist, adamant too that life and inorganic matter were separated by a chasm. Then suddenly in 1800 he switched tracks. His subsequent evolutionary views have always been difficult to interpret (if easy to parody). Corsi explains the origin and real meaning of Lamarck's ideas by tracing their development in detail through the critical period 1800–1802. By this means he is also able to show that, far from accepting spontaneous generation first, as historians had thought, Lamarck only warmed to it after shifting to evolution.

The fact that Lamarck rarely quoted or cited fellow authors has led to the belief that he was acting alone. But again Corsi introduces us to numerous other dissident intellectuals at loggerheads with the powerful Cuvier. Many rejected Cuvier's claim that some fossil animals were extinct, perhaps killed off by a marine

inundation. Like Lamarck, they believed that these animals had simply changed their form in a gradually changing world. As a result, Corsi is able to show that Lamarck's famous *Philosophie Zoologique* (1809) was less an insular pioneering work than a running dialogue with his contemporaries.

Given this context, Lamarck's science makes sense. Of course there were in-

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Still thinking — Lamarck's statue at the entrance to the Jardin des Plantes in Paris.

consistencies. Lamarck had inordinate trouble squaring his progressing animal series with his non-progressionist geology; but, by the 1820s, a new generation had cast aside his old framework and mated evolution to a directionalist Earth history, which provided the environmental motor. There was also a good deal of frustration with the man himself: Lamarck exasperated even sympathizers with his pig-headedness, and they were less and less inclined to come to his aid. Even so, as Corsi says, he was never totally deserted by his friends, any more than he was completely ignored by his enemies, the way the old histories implied.

The Age of Lamarck was first published in Italy in 1983, and this translation (excellent in itself) has been well worth waiting for. Corsi makes Lamarck a man of his time, and that is no mean feat. For historians the result is a treat. But for Lamarck — pilloried and parodied for two centuries — it is long overdue justice. □

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RNA evolution and the origins of life

Gerald F. Joyce

The evolution of RNA is likely to have played an important role in the very early history of life on Earth but it is doubtful that life began with RNA. Consideration of what came before RNA must take into account relevant information from geochemistry, prebiotic chemistry and nucleic acid biochemistry.

THE question of life's origins is one of the oldest and most difficult in biology. The answer, if it is ever known, will not be a single statement of fact but rather an extended chronology, beginning with the formation of the Earth and ending with the appearance of cellular organisms. The problem is especially difficult because we have no direct evidence of the events that occurred during roughly the first thousand million years of the Earth's history. The oldest rocks that provide clues to life's distant past are 3.6×10^9 years old and by that time cellular life seems already to have been well established¹. Modern organisms are so sophisticated that they furnish little information about what life was like before there was a genetic code and a translation apparatus. Extraterrestrial studies have yet to provide us with an alternative life form for comparison. We are left with only a partial understanding of the origins of life that is based largely on inference and conjecture.

From time to time in the quest for such partial answers, a new experimental fact emerges that forces us to view the problem of the origins of life in a different light. The results of the classical Miller-Urey experiments, in which amino acids were produced by circulating H_2 , CH_4 , NH_3 and H_2O past an electric discharge², for example, make it hard to escape the conclusion that amino acids were present on the prebiotic Earth. The synthesis of purines by polymerization of HCN (ref. 3) similarly suggests that adenine, guanine and other purines were available. The most recent example of such a finding is the discovery of RNA molecules with catalytic activity^{4,5}: in addition to their recognized properties as templates, RNA molecules have enzymatic properties and are thus the only molecules known to function as both genotype and phenotype.

This has led to a revival of interest in the idea that there was a time, before the origin of protein synthesis, when life was based entirely on RNA. The idea is not new: it dates back eighteen years before the discovery of catalytic RNA⁶ and was discussed extensively in the late 1960s⁷⁻⁹. But the discovery of catalytic RNA caused molecular biologists to begin to take the notion of an 'RNA world' seriously¹⁰⁻¹². It is by no means a foregone conclusion that life began with the RNA world, although several lines of evidence suggest that the RNA world existed at some stage in evolutionary development. Here, I shall briefly review the advantages of RNA as the basis for the early evolution of life, before discussing the chemical obstacles to the initiation of an RNA world and suggesting how in principle they might have been overcome.

Why RNA?

A fundamental property of living systems is that they have a chemical basis for the storage of genetic information. RNA, DNA and other nucleic-acid-like compounds are informational macromolecules that have inherent template properties and so lend themselves in a straightforward way to both storing information and replicating it. It has been argued that to focus on nucleic acids is to take too parochial a view: there may have been other information storage systems with template properties that were more readily available on the primitive Earth. Cairns-Smith, for example, has proposed¹³ that life began with self-replicating clays, with the genetic information stored as a distribution of charges along a clay surface and replicated by ionic

interactions between existing and newly-formed surface layers. Other templating systems have been proposed based on organic compounds other than nucleic acids^{14,15} or a combination of minerals and associated organics¹⁶. While keeping an open mind about these possibilities, enthusiasm has to be tempered until there is experimental evidence that such an entity can both replicate and code for specific catalytic behaviours.

Could it be that the first genetic molecule did not have template properties and replicated itself in some other way? There was a time when it was widely believed that life began with self-replicating proteins¹⁷. After all, polypeptides are capable of a wide range of catalytic behaviours and it is very likely that they were produced abiotically on the primitive Earth¹⁸. The amino-acid sequence of abiotic polypeptides would have been tremendously heterogeneous, so we must postulate that some mechanism existed to ensure that a particularly useful sequence would facilitate the production of additional copies of itself. Dyson first suggested the possibility of a self-sustaining network of proteins in which the components mutually catalyse the synthesis of each other from monomeric starting materials¹⁹. Kaufmann has argued that, given a large enough assemblage of random polypeptides, the emergence of a self-sustaining network is almost inevitable²⁰. This conclusion seems to me to rest on a highly over-optimistic estimate of the probability that a random polypeptide can catalyse peptide-bond formation with any significant degree of sequence specificity. Any experimental evidence to the contrary would be welcome.

The evidence that life was based on RNA at some stage in evolutionary history is circumstantial but, I believe, persuasive. None of the following points is convincing by itself but together they constitute a body of evidence that cannot be dismissed as coincidental. First, the template properties of RNA enormously simplify the task of self-replication. RNA genomes are known to exist in certain viruses and Orgel has shown that RNA templates are able to direct the synthesis of complementary oligomers in a chemical system²¹. Second, RNA has catalytic properties, as exemplified by the self-splicing activity of certain group I and group II introns^{4,22}, the transfer RNA processing activity of the RNA component of RNase P (ref. 5), and the self-cleaving activity of 'hammerheads' and related RNA structures^{23,24}. These behaviours, although of doubtful prebiotic significance in themselves, indicate that life based on RNA would have included function as well as informational properties. Third, RNA plays an important part in modern biological systems, appearing most conspicuously in those cellular processes that are presumed to be among the most ancient. RNA primes DNA synthesis during DNA replication, carries the genetic message from DNA to the translation apparatus, gathers and positions amino acids in accordance with the codon structure of messenger RNA and functions as an integral part of the ribosome. RNA also has a central role in the spliceosome apparatus^{25,26} and is an essential component of the ribonucleoprotein enzyme that directs telomere synthesis in eukaryotic cells²⁷. Fourth, most of the biological coenzymes are nucleotides or compounds that could be derived from nucleotides (Table 1), a curious fact that may indicate that RNA and RNA enzymes were present before the development of protein synthesis²⁸. Fifth, histidine, which is important in enzyme catalysis as either

a nucleophile or general acid-base catalyst, is produced by a very unusual biosynthetic pathway that begins with phosphoribosyl pyrophosphate and adenosine triphosphate (ATP). The functional imidazole moiety, which now operates as part of a histidine residue within a protein enzyme may once have operated as part of a purine base within an RNA enzyme²⁸. Sixth, in contemporary metabolism, deoxyribonucleotides are synthesized by enzymatic reduction of ribonucleotides rather than by a biosynthetic pathway comparable to that used to synthesize ribonucleotides. The most widely distributed reaction system makes use of three nucleotide-derived components: two molecules of flavin adenine dinucleotide (FAD) associated with thioredoxin, and one molecule of nicotinamide adenine dinucleotide phosphate (NADP). Seventh, deoxythymidylic acid is formed by 5-methylation of deoxyuridylic acid, again suggesting the primacy of ribonucleosides over deoxyribonucleosides.

The most important feature of RNA is that it combines genotype and phenotype in a single molecule, so that replication of RNA enables darwinian evolution to occur at the molecular level. Genetic variation is introduced as a result of mutational errors and RNA-catalysed recombination events. This variation is in turn expressed as a range of phenotypes. Because of differences in their chemical behaviour, some RNAs will replicate more efficiently than others and will grow to dominate the population until a new variant with even greater selective advantage arises²⁹. This is the RNA world.

There are several objections to the notion that life began with RNA, that fall into two general categories. First, RNA is biochemically inept, particularly when compared to proteins whose catalytic prowess is so apparent in modern biochemistry. With the discovery of catalytic RNA, this argument has lost some force but it can still be said that RNA does not seem as versatile a catalyst as protein is. Second, RNA is not a plausible prebiotic

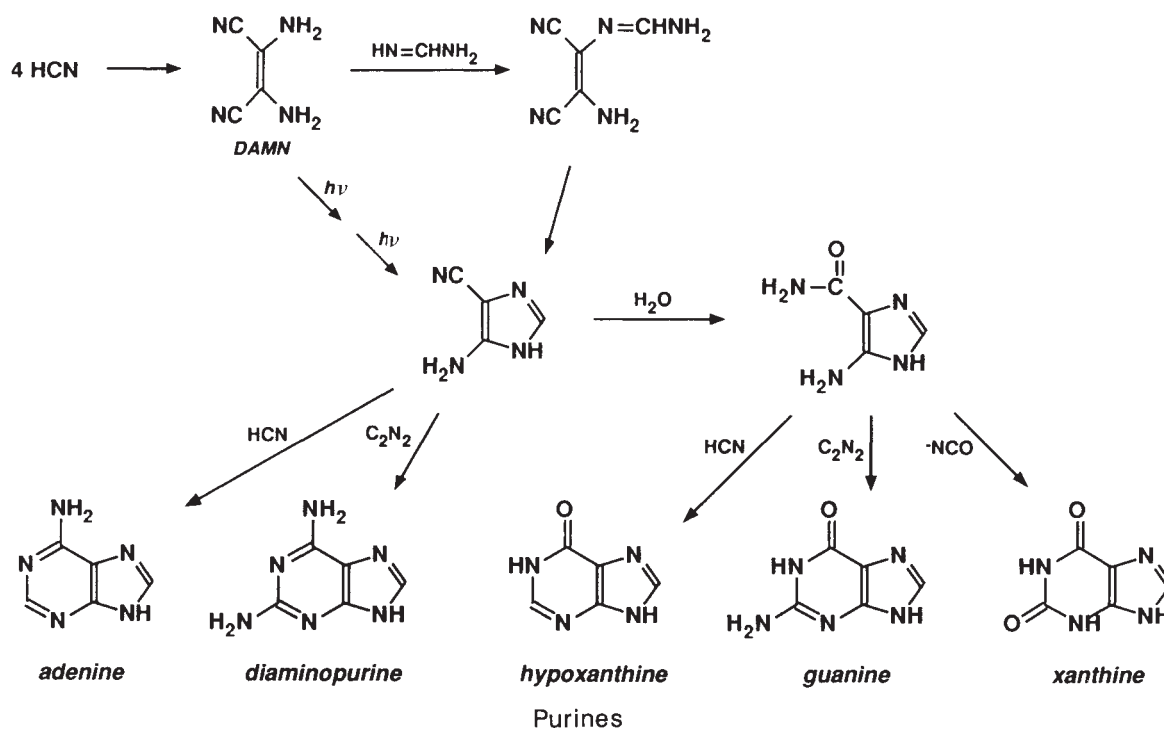


FIG. 1 Prebiotic synthesis of purines by self-condensation of HCN. HCN undergoes a tetramerization reaction to produce diaminomaleonitrile (DAMN). The rate-determining step is the attack on HCN by CN^- to form a dimer,

rapidly converted to a trimer and then to the tetramer, DAMN. This reacts with either formamidine or ultraviolet light to produce 4-aminoimidazole-5-carboxamide, which in turn leads to the formation of various purines.

TABLE 1 Coenzymes that resemble nucleotides

Coenzyme	Base	Sugar-phosphate	Substituent
NAD	Adenine	Ribose 5'-phosphate; ribose 5'-phosphate	Nicotinamide
NADP	Adenine	Ribose 2', 5'-bisphosphate; ribose 5'-phosphate	Nicotinamide
FAD	Adenine	Ribose 5'-phosphate; ribitol 5'-phosphate	Isoalloxazine
FMN	—	Ribitol 5'-phosphate	Isoalloxazine
TPP	Pyrimidine	Pyrophosphate	Hydroxyethylthiazole
THF	2-Amino-4-hydroxy-6-methylpteridine	—	<i>p</i> -aminobenzoic acid; glutamic acid
Coenzyme A	Adenine	Ribose 3'-phosphate, 5'-diphosphate	Pantothenic acid; β -mercaptoethylamine
Coenzyme B ₁₂	Adenine; 5,6-dimethyl-benzimidazole	5'-Deoxyribose; α -ribose 3'-phosphate	Cobalamin
S-adenosylmethionine	Adenine	Ribose	Methionine
ATP	Adenine	Ribose 5'-triphosphate	—
UDP-glucose	Uracil	Ribose 5'-diphosphate	Glucose
CDP-diacylglycerol	Cytosine	Ribose 5'-diphosphate	Diacylglycerol

The carrier molecules S-adenosylmethionine, ATP, UDP-glucose and CDP-diacylglycerol are included with the traditional coenzymes. A number of other nucleoside diphosphate sugars are known to act as carriers in contemporary metabolism. FMN, flavin mononucleotide; TPP, thiamine pyrophosphate; THF, tetrahydrofolate; UDP, uridine diphosphate; CDP, cytidine diphosphate.

molecule because it is unlikely to have been produced in significant quantities on the primitive Earth³⁰. This is a more serious criticism that goes to the heart of the question of whether RNA was the first genetic molecule or whether it took on the role of genetic material at a later stage in evolutionary history. Next, therefore, I consider how difficult it would have been to synthesize RNA in the prebiotic environment.

The primordial setting

Perhaps the most surprising aspect of the origins of life on Earth, other than the fact it happened at all, is that it happened so quickly. The Earth was formed from a diffuse cloud of cosmic gas and dust about 4.6×10^9 years ago³¹ and by most accounts, was a very inhospitable place for the first $0.4\text{--}0.6 \times 10^9$ years of its history. It is virtually certain that no useful organic chemistry could have occurred until about $4.0\text{--}4.2 \times 10^9$ years ago—after the Earth's crust had formed³² and the level of meteoric impacts had subsided so that the environment was no longer in constant upheaval³³. On the other hand, the fossil record strongly supports the existence of cellular life by 3.6×10^9 years ago³⁴ and it has been claimed that indirect evidence exists for biological carbon fixation as early as 3.8×10^9 years ago³⁵. The time window for biogenesis, from organic chemistry to a primitive cell, seems to be 'only' about 0.4×10^9 years.

The composition of the prebiotic atmosphere is a problem of fundamental importance to our understanding of the origins of life. Until recently, it was generally thought that the prebiotic atmosphere was strongly reducing, containing mostly H_2 , CH_4 , NH_3 and H_2O ³⁶. As scientific models of planetary formation have become more sophisticated, it has become increasingly clear that we do not really know whether the prebiotic atmosphere was strongly reducing, mildly reducing or even non-reducing, although it seems certain that there was no free oxygen ($<10^{-6}$ atmospheres) until the advent of oxygen-producing photosynthetic bacteria about $2.5\text{--}2.9 \times 10^9$ years ago³⁷. At present, the weight of evidence indicates that the prebiotic atmosphere was not strongly reducing.

The atmosphere was formed primarily by outgassing of volatile components of the primitive mantle. Its initial composition depends on whether outgassing occurred before or after the iron-rich core of the planet had formed: if outgassing occurred before core formation, contact with metallic iron would have ensured a strongly reducing atmosphere, containing mostly H_2 , H_2O , CH_4 and CO ; if the iron had already sunk to the core, the redox state would have been determined by the $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio in iron minerals, resulting in a less reducing atmosphere that contained H_2O , CO_2 , H_2 and CO but almost no CH_4 ³⁸. It has been argued that even if reduced species such as CH_4 and NH_3 were produced before core formation, they would have been rapidly depleted by photodecomposition³⁹. As time went on, escape of H_2 from the upper atmosphere and outgassing of less reduced species would have tipped the chemical balance in favour of CO and CO_2 over CH_4 and in favour of N_2 over NH_3 . There may have been some mechanism for the continued production of CH_4 and NH_3 , such as the slow re-equilibration of CO_2 and CH_4 at temperatures below 873K (ref. 40), by the arrival of CH_4 and NH_3 on comets and meteorites⁴¹ or by the photochemical reduction of CO_2 in the presence of Fe^{2+} (ref. 42).

Although a strongly reducing environment is most favourable for the synthesis of organic compounds, several prebiotic syntheses have been demonstrated under less strongly reducing conditions. Consider the synthesis of hydrogen cyanide and formaldehyde, which in turn provide a good starting point for the synthesis of more complex materials. Both HCN and H_2CO have been obtained in good yield in spark-discharge experiments under mildly reducing conditions (partial pressure of N_2 of 100 torr and H_2 of 0–400 torr)⁴³. Even in the absence of H_2 , the yield of HCN and H_2CO is about 2% (based on carbon) or about $2\text{--}3 \text{ nmol J}^{-1}$ (based on the energy input). This works out to about $4.5 \times 10^9 \text{ kg yr}^{-1}$ on the primitive Earth, assuming

the energy available from the solar corona discharge was $12.6 \text{ J cm}^{-2} \text{ yr}^{-1}$ (ref. 44). A photochemical source of comparable magnitude has been proposed for both HCN (ref. 45) and H_2CO (ref. 46).

Once produced, HCN would have been hydrolysed and H_2CO would have been destroyed by ultraviolet irradiation, unless they happened to enter a relatively protected microenvironment and managed to combine into more stable compounds. But such combination cannot occur unless the reactants build up to sufficiently high concentrations, which they cannot do unless they are already protected from decomposition. Two possible solutions to this dilemma have been suggested: there may have been a physical mechanism for concentrating the reactants, such as adsorption on a mineral surface⁴⁷ or eutectic freezing in a shallow pond or hail particle⁴⁸; alternatively, the condensation reactions may have been catalysed and thus may have operated at much lower concentrations of reactants. An example is glycolonitrile, which is produced readily from HCN and H_2CO and which enhances the rate of HCN oligomerization by one to two orders of magnitude⁴⁹.

With HCN and H_2CO thus provided, and with the goal of the RNA world in mind, what came next? Assuming that HCN and H_2CO were present in sufficient quantities to kindle HCN oligomerization, several biologically important molecules would

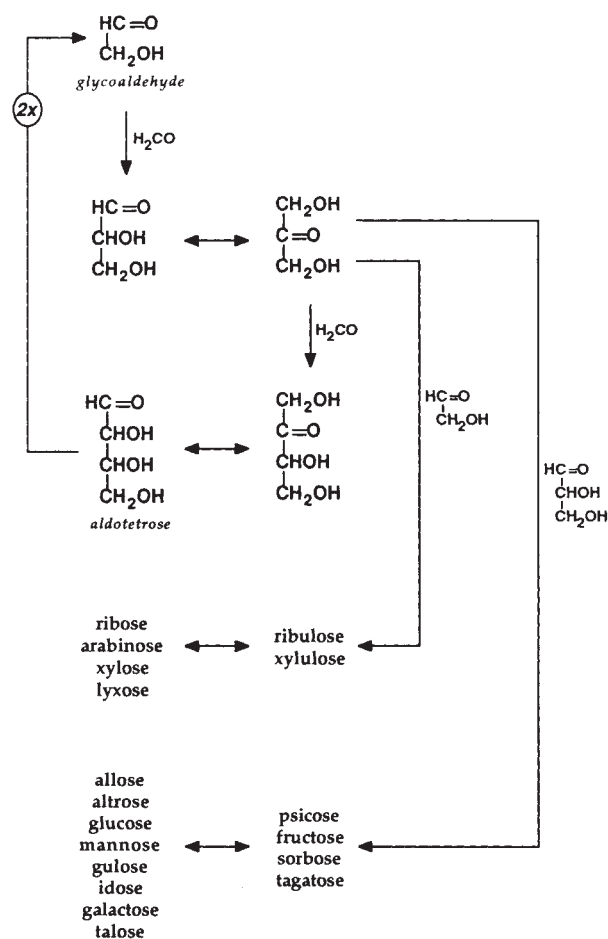


FIG. 2 The formose reaction for the prebiotic synthesis of sugars. Single-headed arrows represent aldol condensations (combination of two aldehydes or ketones to form a β -hydroxyaldehyde or β -hydroxyketone); double-headed arrows represent enolizations (transfer of a proton from an α -carbon to a carbonyl oxygen). The aldotetroses (threose and erythrose) undergo a reverse aldol condensation to yield two molecules of glycolaldehyde. The figure shows D-erythrose as an example of an aldotetrose, but both threose and erythrose can undergo this reaction. Branched-chain and seven-carbon sugars and D- and L-isomers are not shown.

be expected to form (Fig. 1). HCN undergoes a base-catalysed tetramerization reaction to produce diaminomaleonitrile⁵⁰, which participates in several interesting chemical reactions, most notably with formamidine to produce 4-aminoimidazole-5-carbonitrile. This leads to the production of adenine, hypoxanthine, guanine, xanthine and diaminopurine⁵⁰. The 4-aminoimidazole-5-carbonitrile intermediate can also be produced directly from diaminomaleonitrile by irradiating with sunlight, so avoiding the use of formamidine which itself must be derived from HCN and NH₃⁵¹.

Diaminomaleonitrile goes on to form higher oligomers of HCN, which appear as a heterogeneous mixture of compounds. Hydrolysis of HCN oligomers yields glycine, alanine, aspartate, diaminosuccinate and smaller amounts of other amino acids⁵². Amino acids have also been synthesized in spark-discharge experiments performed under mildly reducing conditions⁵³, although the yield and variety of amino acids is substantially lower compared to syntheses performed under strongly reducing conditions. The earliest organisms may have been required to provide their own reducing power, for example using the reduced form of nicotinamide adenine dinucleotide (NAD), NADH, for the biosynthesis of amino acids.

Shifting the focus from HCN to H₂CO, another remarkable polymerization reaction probably took place on the primitive Earth. Accompanying H₂CO would have been smaller amounts of glycoaldehyde, produced either directly or by the condensation of H₂CO in the presence of a catalyst such as calcium carbonate or alumina^{54,55}. Glycoaldehyde begins a cascade of aldol condensations and enolizations that rapidly convert most of the available formaldehyde into trioses, tetroses and higher sugars (Fig. 2). This cascade of reactions, collectively termed the formose (or Butlerow) reaction, proceeds autocatalytically because one molecule of threose or erythrose can be cleaved to give two molecules of glycoaldehyde⁵⁶. From a prebiotic perspective, the formose reaction seems a good way to produce

ribose and other sugars, although unless they were rapidly used to form nucleosides or some other useful compound, they would have been subject to degradation⁵⁷.

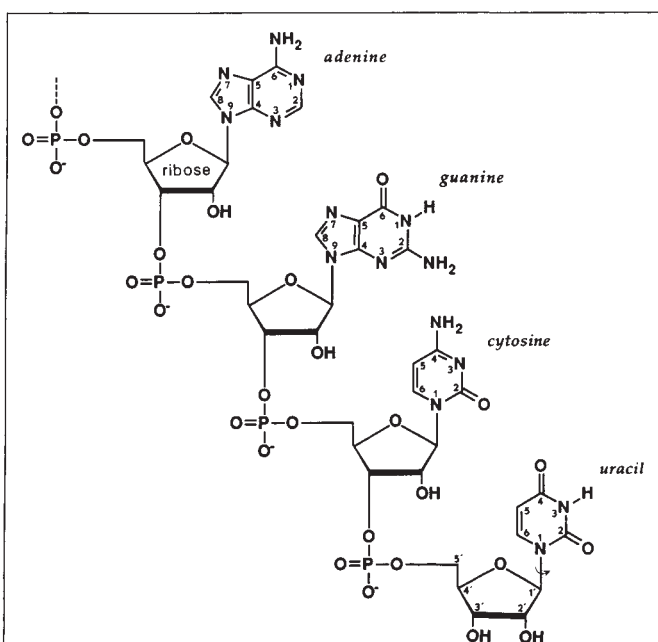
The trouble with RNA

It is a long and difficult road from HCN and H₂CO to the activated nucleoside phosphates that make up RNA (see Box). The self-condensation of HCN to produce purines is such a remarkably simple and efficient reaction that it would be surprising if it did not have some relevance to the early history of life. The prebiotic synthesis of pyrimidines, on the other hand, is more problematic and it is difficult to see how a supply of cytosine or cytosine-containing nucleotides could have been maintained. Cytosine is obtained in about 5% yield when an aqueous solution containing 1.0 M potassium cyanate and 0.1 M cyanoacetylene is incubated at 100 °C for 24 hours⁵⁸. This can hardly be called a prebiotic synthesis because it requires concentrations of reactants that are unlikely to have occurred on the primitive Earth. Cyanoacetylene has been produced by sparking a mixture of CH₄ and N₂ (ref. 58) and presumably could also be obtained from a less reducing mixture. Cyanoacetylene is, however, even more susceptible to hydrolysis than HCN and is unlikely to have built up in significant concentration. There is an alternative route using cyanoacetaldehyde (the first hydrolysis product of cyanoacetylene) and guanidine, although the yields of uracil and, especially, cytosine are low⁵⁹. Furthermore, cytosine would have undergone rapid hydrolysis to uracil (half-life 200 yr at 30 °C)⁶⁰. In contemporary biochemistry, cytidine triphosphate (CTP) is produced by the amination of uridine triphosphate (UTP) in an ATP-dependent reaction.

Another route to the pyrimidines is by acid hydrolysis of HCN oligomers, which can produce 4,5-dihydroxypyrimidine, 5-hydroxyuracil and orotic acid⁶¹. Orotic acid undergoes a photochemical decarboxylation to give uracil⁶², analogous to the enzyme-catalysed decarboxylation that occurs in pyrimidine nucleotide biosynthesis. Uracil can also be produced by condensation of β -alanine and cyanate to give dihydrouracil, followed by photochemical dehydrogenation⁶³. Neither of these pathways for the prebiotic synthesis of pyrimidines is especially convincing.

The greatest problem with the pyrimidines is not their synthesis but the difficulty in attaching them to ribose to form pyrimidine nucleosides. First, consider the synthesis of ribose. The formose reaction may offer a good route for the prebiotic synthesis of sugars but it is unclear how ribose could be singled out from the complex mixture of products (Fig. 2). The five-carbon sugars are produced by condensation of glycoaldehyde and glyceraldehyde, accompanied by comparable amounts of tetroses and hexoses produced by the self-condensation of glycoaldehyde and glyceraldehyde, respectively. Even among the pentoses, there are twelve stereoisomers, eight aldoses and four ketoses, to be reckoned with. Perhaps the chemical properties of ribose led to its selective enrichment by some prebiotic fractionation process. The *cis*-(2,3)-diol structure of ribose and lyxose may have favoured their interaction with a mineral surface where they were relatively protected from hydrolysis compared to arabinose and xylose. The *trans* relationship of the 3-OH and 5-CH₂OPO₃²⁻ groups of ribose-5-phosphate and arabinose-5-phosphate may have rendered them less susceptible to intramolecular cyclization than xylose-5-phosphate and lyxose-5-phosphate⁶⁴. Even if such mechanisms were operating, they would have resulted in a racemic mixture of D- and L-ribose, which still leads to serious difficulties (see below).

Next comes the problem of joining D, L-ribose and a base. When ribose and a purine are heated to dryness in the presence of inorganic salts (Na⁺, Mg²⁺, Ca²⁺, Cl⁻ and SO₄²⁻), a mixture of α - and β -nucleosides is obtained in about 2–10% yield⁶⁵. A comparable reaction between ribose and pyrimidines is very inefficient (<0.1% yield) and no alternative method for the



The primary structure of RNA. Purine and pyrimidine bases are joined to sugar residues (riboses) to form nucleosides. The purines, adenine and guanine, are attached to ribose by a glycosidic bond between C1' of ribose and N9 of the base. The pyrimidines, cytosine and uracil, are attached by a bond between C1' of ribose and N1 of the base. The ribose subunits are joined by a 3', 5' phosphodiester linkage to form the ribose-phosphate backbone. Rotation about the glycosidic bond converts the *anti* conformation to the *syn* conformation. Inversion about C1' would convert the β -conformation to the α -conformation. The numbering schemes for the bases and ribose subunits are shown.

synthesis of pyrimidine nucleosides has been found. In biological systems, the pyrimidines are attached to ribose by displacement of a pyrophosphate at the C1 position but attempts to perform a similar displacement under plausible prebiotic conditions have been unsuccessful⁶⁶. Even the successful synthesis of purine nucleosides is plagued by side reactions, including the production of ribosylamine derivatives based on the C6-amine of adenine or the C2-amine of guanine. In biological systems, the purine nucleosides are synthesized by constructing the purine base on a pre-existing ribose-5-phosphate. A similar synthesis under prebiotic conditions seems far more difficult than using the purines formed by HCN polymerization and joining them directly to ribose.

At this point, the best possibility is a mixture of α , β -D, L-purine nucleosides accompanied by a dizzying array of related compounds. Now comes the task of phosphorylating the nucleosides. Heating a mixture of urea, ammonium chloride and hydroxylapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$] to 100 °C for 24 hours results in a good yield of inorganic polyphosphate⁶⁷. If a nucleoside is added to the mixture, about 20–25% of it is converted to a mixture of nucleoside phosphates, including 2'-, 3'-, 2',3'-cyclic- and 5'-phosphates, and various 2'(3'),5'-diphosphates⁶⁸. Alternative routes using cyanogen or cyanoacetylene rather than high temperature to drive the reaction have been demonstrated⁶⁹, although, as already discussed, these condensing agents were probably not widely available on the primitive Earth.

Finally, the nucleoside phosphates must have been activated to provide a substrate for template-directed polymerization. Adenosine 2', 3'-cyclic phosphate is in a sense already activated; it can be oligomerized by heating it to dryness⁷⁰ or by incubating it at room temperature in the presence of a polyamine⁷¹. Given the proper stereochemical orientation, the 2', 3'-cyclic phosphate undergoes condensation with an adjacent nucleoside 5'-

hydroxyl to produce a 2', 5' or 3', 5' phosphodiester linkage⁷². This is comparable to the RNA-catalysed ligation reaction that has been demonstrated using the satellite RNA of tobacco ringspot virus⁷³ or the genomic RNA of hepatitis delta virus⁷⁴.

A more practical approach might be to activate a nucleoside 5'-phosphate and take advantage of the greater nucleophilicity of the *cis*-(2', 3')-diol compared to the 5'-hydroxyl for the condensation between a 5'-phosphate and 2'(3')-hydroxyl. Adenosine 5'-phosphate can be converted to the diphosphate and subsequently the triphosphate by incubating it with carbamyl phosphate in the presence of a divalent metal cation⁷⁵. Carbamyl phosphate is itself produced by condensation of cyanate and inorganic phosphate in aqueous solution⁷⁶. Nucleoside 5'-polyphosphates have also been obtained by heating a solution of nucleotides and inorganic polyphosphate to dryness⁷⁷.

Could this long and difficult road to activated mononucleotides be travelled without biological catalysts (or an organic chemist) to lead the way? Suppose that somehow it did happen; that given a few hundred million years and an entire globe of microenvironments, there was a special time and place in which a rich soup of activated mononucleotides formed. The only flaw in this hypothetical garden of Eden is that the soup would contain a racemic mixture of D- and L-nucleotides, a necessary consequence of the fact that the prebiotic environment was achiral, which, it turns out, makes RNA replication very difficult. By a strange twist of nucleotide chemistry, D-mononucleotides in the *anti* conformation and L-mononucleotides in the *syn* conformation tend to form very close structural homologues when bound to a complementary template⁷⁸ (Fig. 3). As a result, the template-directed synthesis of one enantiomer is strongly inhibited by the presence of the other⁷⁹. The 'wrong' enantiomer adds to the 3' end of a growing chain, which distorts the 3' terminus and thus prevents further chain elongation.

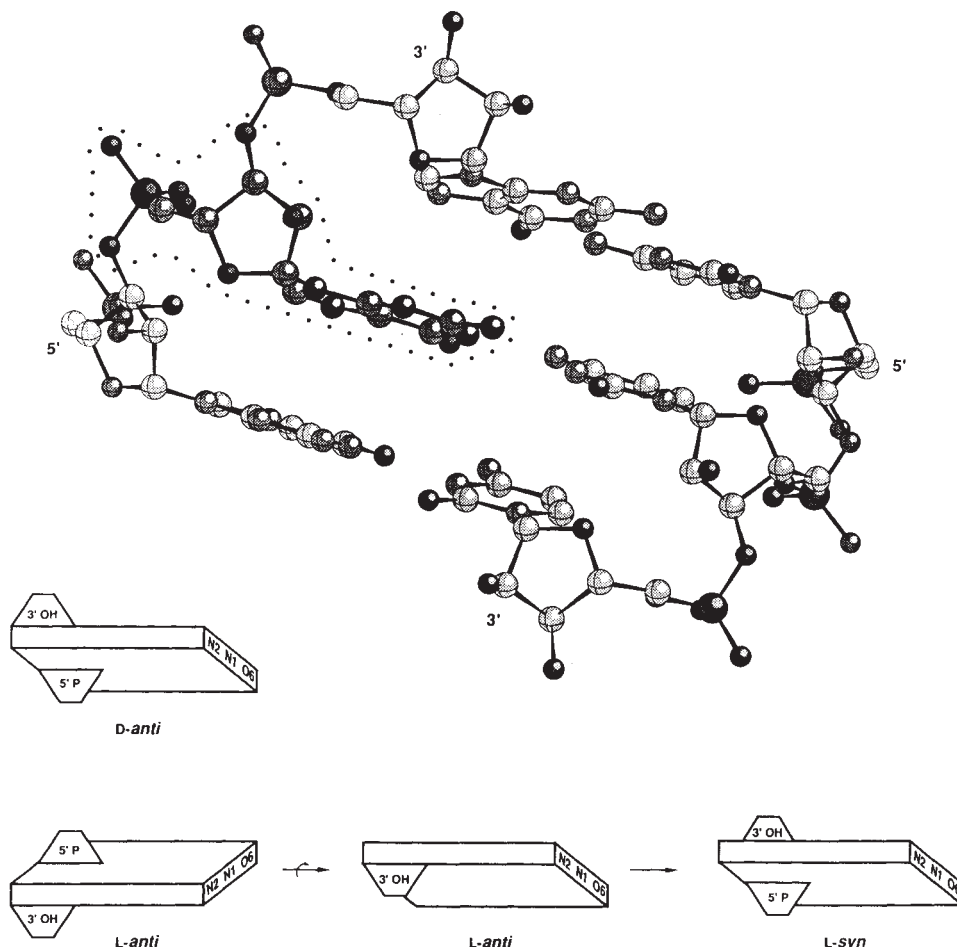


FIG. 3 Top, three successive guanine-cytosine pairs of the A-form of RNA projected onto a plane perpendicular to the diad axis. The middle guanosine residue is enclosed in a dotted box. Atom coordinates are based on the crystal structure of A-RNA⁹⁶. Graphical representation was performed using *Ball & Stick* software on a Macintosh computer. Bottom, the close structural homology between D-*anti*- and L-*syn*-guanosine mononucleotides when bound to a complementary template. The D-*anti* form corresponds to the middle guanosine residue shown above. Imagine reflecting this in a mirror to produce the L-*anti* form, which is rotated about its long axis to orient the base for complementary pairing and finally rotated about the glycosidic bond to produce the L-*syn* form.

If one admits both α - and β -nucleotides to the hypothetical garden of Eden, another cross-inhibition phenomenon is likely to occur. α -D-mononucleotides in the *syn* conformation and β -D-mononucleotides in the *anti* conformation also form close structural homologues⁸⁰ and any combination of α -D-*syn*-, α -L-*anti*-, β -D-*anti*-, or β -L-*syn*-mononucleotides is likely to be a problem. The addition of arabinose, other pentoses and the hexoses is an open invitation to template-directed chaos. It is difficult to imagine how a genetic message could be transferred from template to complementary product in the face of all this isomeric interference. The most reasonable interpretation is that life did not start with RNA. The RNA world came into existence after many of the problems associated with the prebiotic synthesis and template-directed replication of RNA had been solved. This implies that there was a simpler genetic system, or systems, that preceded RNA and that evolutionary advances made by the ancestral system were somehow carried over to the RNA world.

The origin of self-replication

We know very little about the chemical nature of the first self-replicating molecule. There are four general requirements for the primordial genetic material, regardless of its chemical nature. First, it must have had informational properties, suggesting that it was some type of heteropolymer, composed of at least two monomeric subunits. Second, it must have been capable of directing the ordered assembly of monomeric starting materials to form additional copies of itself. Third, the monomeric starting materials must have been readily available, at least in some locale on the primitive Earth. Fourth, the genetic material must have been sufficiently stable for its rate of reproduction to exceed its rate of decomposition. If the goal is the eventual origin of an RNA world, then a requirement for an evolutionary pathway from the first self-replicating molecule to a genetic system based on RNA must be added.

There are three ways in which the difficulties in arriving at an RNA world may have been reduced. One is that before the origin of self-replication, there was a period of chemical evolution⁸¹, during which non-instructed processes led to progressive alteration of the chemical composition of the environment. The prebiotic environment can be thought of as a flow reactor, driven by the supply of HCN, H₂CO and other high-energy starting materials, and characterized by a steady-state distribution of reactants and products. In this context, the formose reaction for instance, may not have been quite so unruly; rather than the cascade of all possible 3-, 4-, 5-, 6- and 7-carbon sugars obtained from batch polymerization of H₂CO, there might have been more limited distribution of sugars determined by the differential rates of formation and decomposition of the various compounds⁸². Non-instructed chemical ordering may have led to very complex structures, such as colloidal aggregates of polypeptides¹⁸ and even self-propagating membranous vesicles⁸³. These complex structures may have had a profound influence on the local environment and may have set the stage for the emergence of a self-replicating system.

A second possibility is the intervention of a primitive self-replicating system chemically unrelated to RNA, which may have helped to bias the environment in a way that made the appearance of RNA or RNA-like molecules more likely. The most carefully constructed hypothesis along these lines concerns the possibility of self-replicating clay minerals¹³, where the genetic material is considered to be a distribution of ionic charges, embedded organic compounds or structural defects along a mineral surface that serves as a template for the formation of additional mineral layers. At one extreme, these clay 'organisms' may have come and gone, leaving their mark by altering the local environment. At the other extreme, they may have developed the capacity to synthesize RNA or RNA-like molecules that were used initially as catalysts or cofactors in the clay world but eventually themselves became self-replicating and took on the role of genetic material⁸⁴.

The third advance on the way to an RNA world may have been the appearance of a self-replicating chemical system similar to RNA but simpler in the sense that it demanded less from the chemistry of the prebiotic environment^{78,85}. For example, the difficulties associated with the prebiotic synthesis of pyrimidines could be circumvented if the genetic material were based on purines alone. Non-standard pairing combinations such as adenine: inosine, adenine:xanthine and guanine:isoguanine have been suggested^{8,86}, although there is no experimental evidence that they are suitable for template-directed polymerization. The backbone might have been simpler, with ribose replaced by something that was not accompanied by dozens of closely related compounds or subject to enantiomeric cross-inhibition. A number of flexible, acyclic nucleoside analogues that fit this description have been proposed^{78,87} (Fig. 4).

The compound that has been studied in the greatest detail is the glycerol-derived nucleoside analogue (compound *b* in Fig. 4). In principle, it could be synthesized by condensation of H₂CO and glycerol followed by addition of a base. This should produce a mixture of various mono- and di-substituted glycerol derivatives but this would be far simpler than the assortment of ribosyl- and other sugar derivatives that would accompany a nucleoside. This compound, either non-phosphorylated or phosphorylated at both hydroxyl positions, is an achiral molecule. It becomes chiral when incorporated into a heteropolymeric structure. The glycerol-derived analogue of guanosine bisphosphate, when activated at both phosphates, undergoes a poly(C)-directed oligomerization reaction to form pyrophosphate-linked products⁸⁸. A pyrophosphate-linked oligomer formed from deoxycytidine bisphosphate has also been used as a template to direct the oligomerization of deoxyguanosine bisphosphate derivatives (A. W. Schwartz, personal communication). Thus there could have been a genetic molecule with a glycerol-phosphate backbone that replicated by a chemical process and was able to undergo darwinian evolution.

Chemical self-replication, in the glycerol-phosphate system or in any RNA-like system, faces several obstacles. One of the most serious is the need for end-to-end copying of the template before the development of a mechanism to ensure that replica-

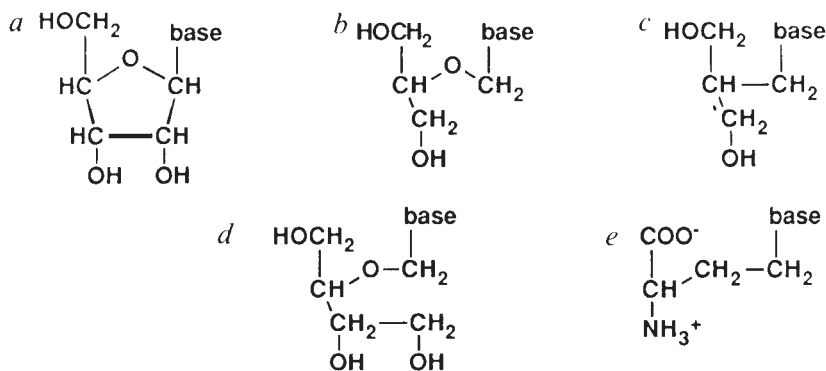


FIG. 4 Comparative structure of a nucleoside and of four simple acyclic nucleoside analogues. *a*, nucleoside; *b*, glycerol-derived acyclonucleoside; *c*, acrolein-derived nucleoside analogue; *d*, erythritol-derived acyclonucleoside; *e*, aspartate-derived nucleoside analogue.

tion begins at a discrete initiation site. In the RNA world this problem may have been solved by a 'genomic-tag' structure that directed a primitive replicase to the appropriate initiation site⁸⁹. In the early stages of self-replication, a chemical tag could have demarcated one end of the template or a chemical ligation mechanism could have joined partial copies together to form complete products.

Another important obstacle to chemical self-replication, which has been demonstrated in experimental systems, is the tendency of molecules with regions of self-complementarity to form intramolecular duplex structures⁹⁰. It is difficult to envisage a solution to this problem in the absence of a factor such as single-strand binding proteins, because the conditions must favour short duplex regions so that template-directed synthesis can occur yet disfavour long duplex regions that would produce stable secondary structures. There may be alternative helical backbones that have non-cooperative properties so that longer duplex structures are increasingly disfavoured⁷⁸. In any event, the development of an unwinding activity would have been a top priority during early evolutionary history.

Finally, there is the problem of accurate copying of the genetic information in the absence of a sophisticated replicase enzyme. The selectivity of complementary base pairing provides the chemical basis for information transfer, with the fidelity depending on the particular bases involved (highest for cytosine→guanine, lowest for adenine→uracil)⁹¹. The efficiency of copying also depends on base sequence, with selection pressure favouring those sequences that can be copied most readily. One of the tasks of a polymerase enzyme is to 'smooth' these differences so that a broader range of sequences can be explored⁹². But given the limits on genome size (as determined by copying fidelity²⁹) and on sequence heterogeneity, would there have been enough potential sequences remaining to evolve a smoothing function that could further expand the range of potential sequences? A crude replicase activity must have arisen so that copying fidelity could be improved, genome size could be increased and ever more sophisticated catalysts could be developed.

Transition to an RNA world

Let us suppose that after a period of chemical evolution, with or without further augmentation by a genetic system completely unrelated to RNA, a genetic system arose based on some simple RNA-like molecule. It is not inconceivable that a genetic system completely unrelated to RNA would have invented RNA for its own purposes. With so many design problems associated with RNA synthesis, it seems more likely that a simple RNA-like molecule was invented instead. Once a genetic system based on an RNA-like molecule existed, the domain of nucleotide chemistry would have been more accessible and the invention of RNA would have been more practical. Why should an increasingly sophisticated RNA-like world come to synthesize mononucleotides rather than optimize the synthesis of its own monomeric units? The answer must be that mononucleotides somehow conferred a selective advantage to the RNA-like world, either by improving its information storage properties or by enhancing its catalytic abilities.

Reviewing the catalytic properties of known RNA enzymes⁹³,

RNA catalysis seems to be limited to sequence-specific cleavage/ligation reactions involving nucleic acid substrates. But the chemical properties of the various functional groups within RNA suggest a broader range of catalytic function (Table 2). Of particular importance to RNA, but not to any of its likely predecessors, is the ribose 2' hydroxyl. A terminal *cis*-(2', 3')-diol is a good nucleophile and an internal 2'-hydroxyl can function either as a nucleophile²² or as a determinant of RNA tertiary structure^{94,95}. Because of the 2'-hydroxyl, RNA may have enhanced the structural and catalytic properties of the RNA-like world and its production could have been a favourable event.

We do not know how the transition from an RNA-like to an RNA world was made. There are several ways in which RNA may have gained entry. One is that nucleotides or nucleotide derivatives were generated to perform some useful function in the RNA-like world and then took root as cofactors in primitive metabolic processes. The RNA cofactors progressed from monomeric to oligomeric forms and eventually became autonomously self-replicating. Another possibility is that mononucleotides were at first inadvertently incorporated into the ancestral genetic material but this proved selectively advantageous. It is not clear how random misincorporations could have been useful or how the incorporation of mononucleotides could have been limited to specific sites. Somehow the genetic material took on more and more of the character of RNA without forsaking its past evolutionary accomplishments. A third possibility is that mononucleotides, originally present as cofactors, became oligomerized in a template-dependent way to give products that contained only RNA components. The ancestral genetic material directed both its own replication and the 'transcription' of RNA. RNA became autonomously self-replicating and either shed its RNA-like predecessor or, through reverse transcription, replaced the predecessor with DNA.

The transition to an RNA world, like the origins of life in general, is fraught with uncertainty and is plagued by a lack of relevant experimental data. Researchers into the origins of life have grown accustomed to the level of frustration in these problems. Perhaps it is time for a new group of scientists to join in the frustration. The discovery of catalytic RNA and the appreciation of the dual role of RNA as both genetic material and catalyst have kindled new interest in the evolution of RNA and the origins of life. It is time to go beyond talking about an RNA world and begin to put the evolution of RNA in the context of the chemistry that came before it and the biology that followed. □

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TABLE 2 Catalytic potential of RNA

Component	Metal-ion coordination	General acid catalysis	General base catalysis	Nucleophile
Ribose	2'OH			2'OH, 2'(3')-diol
Phosphate	O ⁻	OH	O ⁻	
Adenine	N7		N1	
Guanine	N7	N1	N7	
Cytosine	N3		N3	
Uracil	O2	N3		

- Walter, M. R. in *Earth's Earliest Biosphere* (ed Schopf, J. W.) 187–213 (Princeton University Press, 1983).
- Miller, S. L. *Science* **117**, 528–529 (1953).
- Oró, J. *Nature* **191**, 1193–1194 (1961).
- Kruger, K. et al. *Cell* **31**, 147–157 (1982).
- Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N. & Altman, S. *Cell* **35**, 849–857 (1983).
- Quastler, H. *The Emergence of Biological Organization* Ch. 1 (Yale University Press, New Haven, 1964).
- Woese, C. *The Genetic Code* Ch. 7 (Harper & Row, New York, 1967).
- Crick, F. H. C. *J. molec. Biol.* **38**, 367–379 (1968).
- Orgel, L. E. *J. molec. Biol.* **38**, 381–393 (1968).
- Sharp, P. A. *Cell* **42**, 397–400 (1985).
- Lewin, R. *Science* **231**, 545–546 (1986).
- Gilbert, W. *Nature* **319**, 618 (1986).
- Cairns-Smith, A. G. *J. theor. Biol.* **10**, 53–88 (1966).
- Nelsestuen, G. L. *J. molec. Evol.* **15**, 59–72 (1980).
- Weber, A. L. *Origins of Life* **17**, 107–119 (1987).
- Orgel, L. E. *Origins of Life* **17**, 27–34 (1986).
- Oparin, A. I. *Adv. Enzymol.* **27**, 347–380 (1965).
- Fox, S. W. & Dose, K. *Molecular Evolution and the Origin of Life* (Dekker, New York, 1977).
- Dyson, F. J. *J. molec. Evol.* **18**, 344–350 (1982).
- Kauffman, S. A. *J. theor. Biol.* **119**, 1–24 (1986).
- Inoue, T. & Orgel, L. E. *Science* **219**, 859–862 (1983).
- Peebles, C. L., Mecklenberg, K. L., Perlman, P. S., Tabor, J. & Cheng, H. L. *Cell* **44**, 213–223 (1986).
- Uhlenbeck, O. C. *Nature* **328**, 596–600 (1987).
- Sharmeen, L., Kuo, M. Y. P., Dinter-Gottlieb, G. & Taylor, J. J. *Viol.* **62**, 2674–2679 (1988).
- Sharp, P. A. *Science* **235**, 766–771 (1987).
- Maniatis, T. & Reed, R. *Nature* **325**, 673–678 (1987).
- Greider, C. W. & Blackburn, E. H. *Cell* **51**, 887–898 (1987).

28. White, H. B. *J. molec. Evol.* **7**, 101-104 (1976).
29. Eigen, M. *Naturwiss.* **58**, 465-523 (1971).
30. Shapiro, R. *Origins of Life* **14**, 565-570 (1984).
31. Papanastassiou, D. A. & Wasserburg, G. J. *Earth planet. Sci. Lett.* **11**, 37-62 (1971).
32. Ernst, W. G. in *Earth's Earliest Biosphere* (ed Schopf, J. W.) 41-52 (Princeton University Press, 1983).
33. Maher, K. A. & Stevenson, D. J. *Nature* **331**, 612-614 (1988).
34. Buick, R., Dunlop, J. S. R. & Groves, D. I. *Alcheringa* **5**, 161-181 (1981).
35. Schidlowski, M., Appel, P. W. U., Eichmann, R. & Junge, C. E. *Geochim. Cosmochim. Acta* **43**, 189-199 (1979).
36. Dickerson, R. E. *Sci. Am.* **239**, 70-86 (1978).
37. Hayes, J. M. in *Earth's Earliest Biosphere* (ed Schopf, J. W.) 291-301 (Princeton University Press, 1983).
38. Chang, S., DesMarais, D., Mack, R., Miller, S. L. & Strathearn, G. E. in *Earth's Earliest Biosphere* (ed Schopf, J. W.) 53-92 (Princeton University Press, 1983).
39. Levine, J. S. & Augustsson, T. R. *Origins of Life* **15**, 299-318 (1985).
40. Deines, P. *Geochim. cosmochim. Acta* **44**, 943-961 (1980).
41. Oró, J. *Nature* **190**, 389-390 (1961).
42. Borowska, Z. & Mauzerall, D. *Proc. natn. Acad. Sci. U.S.A.* **85**, 6577-6580 (1988).
43. Stribling, R. & Miller, S. L. *Origins of Life* **17**, 261-273 (1987).
44. Miller, S. L. & Urey, H. C. *Science* **130**, 245-251 (1959).
45. Zahnle, K. J. *J. geophys. Res.* **91**, 2819-2834.
46. Pinto, J. P., Gladstone, G. R. & Young, Y. L. *Science* **210**, 183-185 (1980).
47. Bernal, J. D. *The Physical Basis of Life* **34** (Routledge & Kegan Paul, London, 1951).
48. Sanchez, R., Ferris, J. & Orgel, L. E. *Science* **153**, 72-73 (1966).
49. Schwartz, A. W. & Goverde, M. J. *molec. Evol.* **18**, 351-353 (1982).
50. Sanchez, R. A., Ferris, J. P. & Orgel, L. E. *J. molec. Biol.* **30**, 223-253 (1967).
51. Ferris, J. P. & Orgel, L. E. *J. Am. Chem. Soc.* **88**, 1074 (1966).
52. Ferris, J. P. & Hagan, W. J. *Tetrahedron* **40**, 1093-1120 (1984).
53. Schlesinger, G. & Miller, S. L. *J. molec. Evol.* **19**, 376-382 (1983).
54. Gabel, N. W. & Ponnampuram, C. *Nature* **216**, 453-455 (1967).
55. Reid, C. & Orgel, L. E. *Nature* **216**, 455 (1967).
56. Mizuno, T. & Weiss, A. H. *Adv. Carbohydr. Chem. Biochem.* **29**, 173-227 (1974).
57. Mopper, K., Dawson, R., Liebezeit, G. & Ittekkot, V. *Mar. Chem.* **10**, 55-66 (1980).
58. Sanchez, R. A., Ferris, J. P. & Orgel, L. E. *Science* **154**, 784-785 (1966).
59. Ferris, J. P., Zamek, O. S., Altbuch, A. M. & Freiman, H. J. *molec. Evol.* **3**, 301-309 (1974).
60. Shapiro, R. & Klein, R. S. *Biochemistry* **5**, 2358-2362 (1966).
61. Ferris, J. P., Joshi, P. C., Edelson, E. H. & Lawless, J. G. J. *molec. Evol.* **11**, 293-311 (1978).
62. Ferris, J. P. & Joshi, P. C. *J. Org. Chem.* **44**, 2133-2137 (1979).
63. Chittenden, G. J. F. & Schwartz, A. W. *Nature* **263**, 350-351 (1976).
64. Hill, A. R., Nord, L. D., Orgel, L. E. & Robins, R. K. *J. molec. Evol.* **28**, 170-171 (1988).
65. Fuller, W. D., Sanchez, R. A. & Orgel, L. E. *J. molec. Biol.* **67**, 25-33 (1972).
66. Orgel, L. E. & Lohrmann, R. *Accounts Chem. Res.* **7**, 368-377 (1974).
67. Osterberg, R., Orgel, L. E. & Lohrmann, R. *J. molec. Evol.* **2**, 231 (1973).
68. Lohrmann, R. & Orgel, L. E. *Science* **171**, 490-494 (1971).
69. Lohrmann, R. & Orgel, L. E. *Science* **161**, 64-66 (1968).
70. Bishop, M. J., Lohrmann, R. & Orgel, L. E. *Nature* **237**, 162-164 (1972).
71. Verlender, M. S., Lohrmann, R. & Orgel, L. E. *J. molec. Evol.* **2**, 303-316 (1973).
72. Usher, D. A. *Nature New Biology* **235**, 207 (1972).
73. Buzayan, J. M., Gerlach, W. L. & Bruening, G. *Nature* **323**, 349-353 (1986).
74. Wu, H.-N. & Lai, M. M. C. *Science* **243**, 652-654 (1989).
75. Saygin, O. *Naturwiss.* **67**, 617-619 (1981).
76. Jones, M. E. & Lipmann, F. *Proc. natn. Acad. Sci. U.S.A.* **46**, 1194-1205 (1960).
77. Lohrmann, R. *J. molec. Evol.* **6**, 237-252 (1975).
78. Joyce, G. F., Schwartz, A. W., Miller, S. L. & Orgel, L. E. *Proc. natn. Acad. Sci. U.S.A.* **84**, 4398-4402 (1987).
79. Joyce, G. F. *et al. Nature* **310**, 602-604 (1984).
80. Praseuth, D. *et al. J. molec. Biol.* **196**, 939-942 (1987).
81. Calvin, M. *Chemical Evolution* (Oxford University Press, New York, 1969).
82. Harsch, G., Harsch, M., Bauer, H. & Voelter, W. *Z. Naturforsch.* **38b**, 1269-1280 (1983).
83. Morowitz, H. J., Heinz, B. & Deamer, D. *Origins of Life* **18**, 281-287 (1988).
84. Cairns-Smith, A. G. in *Origin of Life: Proceedings of the Second ISSOL Meeting, Kyoto, Japan* (ed Noda, H.) 399-404 (Center for Academic Publications, Tokyo, 1978).
85. Spach, G. *Origins of Life* **14**, 433-437 (1984).
86. Wächtershäuser, G. *Proc. natn. Acad. Sci. U.S.A.* **85**, 1134-1135 (1988).
87. Orgel, L. E. in *Evolutionary Tinkering in Gene Expression* (ed Grunberg-Manago, M.) (Plenum, London, in the press).
88. Schwartz, A. W. & Orgel, L. E. *Science* **228**, 585-587 (1985).
89. Weiner, A. M. & Maizels, N. *Proc. natn. Acad. Sci. U.S.A.* **84**, 7383-7387 (1987).
90. Joyce, G. F. & Orgel, L. E. *J. molec. Biol.* **188**, 433-441 (1986).
91. Joyce, G. F. *Cold Spring Harbor Symp. quant. Biol.* **52**, 41-51 (1987).
92. Joyce, G. F., Inoue, T. & Orgel, L. E. *J. molec. Biol.* **176**, 279-306 (1984).
93. Cech, T. R. *Science* **236**, 1532-1539 (1987).
94. Pace, N. R. & Marsh, T. L. *Origins of Life* **16**, 97-116 (1985).
95. Dock-Bregeon, A. C. *et al. Nature* **335**, 375-378 (1988).
96. Arnott, S. & Hukins, D. W. L. *Biochem. Biophys. Res. Commun.* **48**, 1392-1399 (1972).

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Structure of tumour necrosis factor

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Tumour necrosis factor is a trimeric molecule, each subunit of which consists of an antiparallel β -sandwich. Individual subunits form the trimer by a novel edge-to-face packing of β -sheets. A comparison of the subunit fold with that of other proteins reveals a remarkable similarity to the 'jelly-roll' structural motif characteristic of viral coat proteins.

TUMOUR necrosis factor (TNF) plays a key role as a polypeptide mediator of inflammation and the cellular immune response. It is secreted by macrophages and is a member of the class of molecules termed cytokines, a group which includes the interferons and interleukins. These hormones form a complex network of interactive signals that regulate their own production and the growth and differentiation of cells involved in inflammation, immunity and haemopoiesis¹. TNF was first characterized by its *in vivo* antitumour properties and *in vitro* cytotoxicity to certain transformed cell lines². Lipopolysaccharide (LPS) is a potent trigger for TNF production^{3,4} and the effects of LPS such as fever and shock seem to be mediated by TNF. TNF also displays antiviral activity^{5,6} and plays a protective role in malarial infections⁷. Conversely, it is implicated in the pathogenesis of cachexia (wasting)⁴, rheumatoid arthritis and inflammatory tissue destruction^{8,9}.

TNF is secreted as an unglycosylated 157-amino-acid polypeptide of relative molecular mass (M_r) 17,350 (17 K). Its primary structure is evolutionarily conserved¹⁰, and in its active form the protein is thought to be trimeric¹¹. TNF binds to a receptor, believed to be a glycoprotein of $M_r \sim 75$ K¹¹⁻¹⁷.

We report here the determination of the structure of the TNF trimer at 2.9 Å resolution by X-ray crystallography. The main-chain fold of a TNF subunit shows a remarkable similarity to the 'jelly-roll' structural motif of viral capsid proteins, making it unique among known non-viral proteins.

Structure determination

Material of high purity was obtained from the recombinant human TNF gene expressed in *Escherichia coli* as the 17 K protein. Crystals of TNF grew by vapour diffusion as hexagonal rods (0.5 × 0.5 × 1.5 mm) from ammonium sulphate solution at room temperature (N.P.C.W. and S. Marcinowski, unpublished observations). The crystals belong to the trigonal space group $P3_121$ with unit cell dimensions $a = b = 166$ Å, $c = 93$ Å and diffract to 2.9 Å resolution. Crystal density measurements using the Ficoll density gradient method indicated that the asymmetric unit probably contained two trimers; the crystal solvent content was therefore 65%.

Data were generated and collected using synchrotron radiation at the SERC Daresbury Laboratory and a Xenonics multiwire area detector at Oxford (Table 1). The presence of six subunits of unknown orientation within the asymmetric unit made the solution of heavy atom derivatives problematic; self-rotation function calculations failed to locate the non-crystallographic 3-fold axes of the two trimers. Analysis of difference Patterson maps for putative heavy-atom derivatives was undertaken using programs (GROPAT) written specifically for this project (E.Y.J. and D.I.S., manuscript in preparation). This analysis provided the heavy atom positions for an $\text{Hg}(\text{CH}_3\text{CO}_2)_2$

derivative that had one heavy-atom site per subunit. Difference Fouriers then revealed two other usable multiple site derivatives of poorer quality. Phasing statistics are given in Table 1.

Solvent flattening was applied to the three 6 Å SIR maps for these derivatives, in turn, and a 'best' map was then produced as a weighted average of these. From this starting point, gradual and very careful phase extension to 4 Å was carried out using phase information from the first derivative combined with solvent flattening. Beyond 4 Å no phase information was available from the heavy-atom derivatives. Further phase extension was achieved by solvent flattening and averaging using the Bricogne algorithms¹⁹. We performed 3-fold averaging separately about the two trimers. After initial cycling at 4 Å, very gradual phase extension to the limit of the native data, 2.9 Å, was carried out. Although the positions of both non-crystallographic 3-fold axes refined satisfactorily, one of the two trimers (the second) showed significantly poorer averaging statistics. After cycling to convergence at 2.8 Å, it was decided to remove the constraint of 3-fold averaging for the second trimer and continue with this modified cycle of partial averaging and solvent flattening. Surprisingly, this gave electron density of an improved quality for both trimers. Averaging and phase extension statistics are given in Table 1.

An unambiguous chain trace was quickly established for one of the three subunits of trimer one, and confirmed by fitting the amino-acid sequence. Apart from two regions of high mobility there are no main-chain breaks, side-chain density is well defined, and a high percentage of main-chain carbonyl bulges are visible. A representative portion of the map is shown in Fig. 1. The final model comprises 153 residues and is complete but for the first 4 N-terminal amino acids for which clear electron density is not visible. The structure has now been refined using X-PLOR²⁰ on a CONVEX C210. The *R*-factor is at present 22.2% (on all data from 6 to 2.9 Å) after restrained *B*-factor refinement with excellent stereochemistry (r.m.s. deviation from

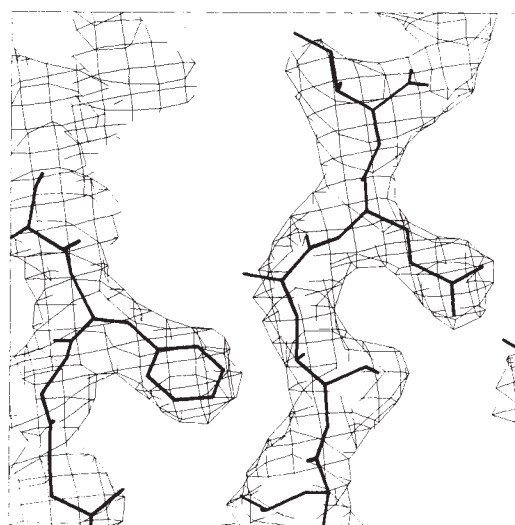


FIG. 1 Portion of the electron-density map (before refinement) viewed edge-on to the β -sandwich. Phe 152 in β -strand I and Glu 135 in β -strand H are shown in the centre of the figure. Electron density contoured at an arbitrary level is represented by chicken wire.

ideal bond lengths of 0.013 Å) and no model for the solvent structure.

Description of the structure

TNF is a compact trimer composed of 3 identical subunits of 157 amino acids (Fig. 2). The overall shape of the monomeric subunit is wedge-like with length 50 Å and maximum breadth 30 Å. It is essentially a β -sandwich structure formed by two antiparallel β -pleated sheets and has the topology of the classic viral jelly-roll motif (Fig. 3a). The upper sheet (sheet 1) is kinked with three long strands supplemented for half the sheet by a loop of two additional strands. The lower sheet (sheet 2) comprises five strands of steadily decreasing length. The middle strand of this sheet consists of the last nine C-terminal residues; the C terminus is thus firmly locked into position. By contrast, the flexible N-terminal region runs past a neighbouring subunit in the trimer before the chain is incorporated into the protein core as a β -strand in sheet 2. It has previously been concluded⁴ that the complete carboxy-terminal sequence is essential for bioactivity, whereas amino-terminal deletions of up to eight residues may be sustained without loss of bioactivity. This is consistent with the maintenance of structural integrity in sheet

2. The single disulphide bridge (residues 69 and 101) stretches between the two longest intersheet loops. This region (in particular residues 102-113 in the larger of these loops) comprises the other area of high main-chain mobility.

The subunits associate tightly about the 3-fold to form a compact conical-shaped trimer of length 50 Å and maximum breadth 45 Å. The interaction is through a simple edge-to-face packing of the β -sandwich (Fig. 2b). The edge of the β -sandwich consisting of strands F and G from one subunit lies across sheet 2 of a 3-fold related subunit. The carboxy terminus lies close to the 3-fold axis. The subunit β -sandwich has the expected filling of tightly intercalating large apolar residues. The outer surface of the trimer, mainly comprising the outside of sheet 1, is decorated with charged and polar side-chains. The remaining face, that of sheet 2 which mediates the 3-fold edge-to-face interaction, is predominantly polar in character.

Structural homology

The connectivity for the standard eight strands of the viral jelly-roll motif and the equivalent elements of secondary structure in the TNF subunit is identical. A series of comparisons between TNF and other β -barrel proteins has been carried

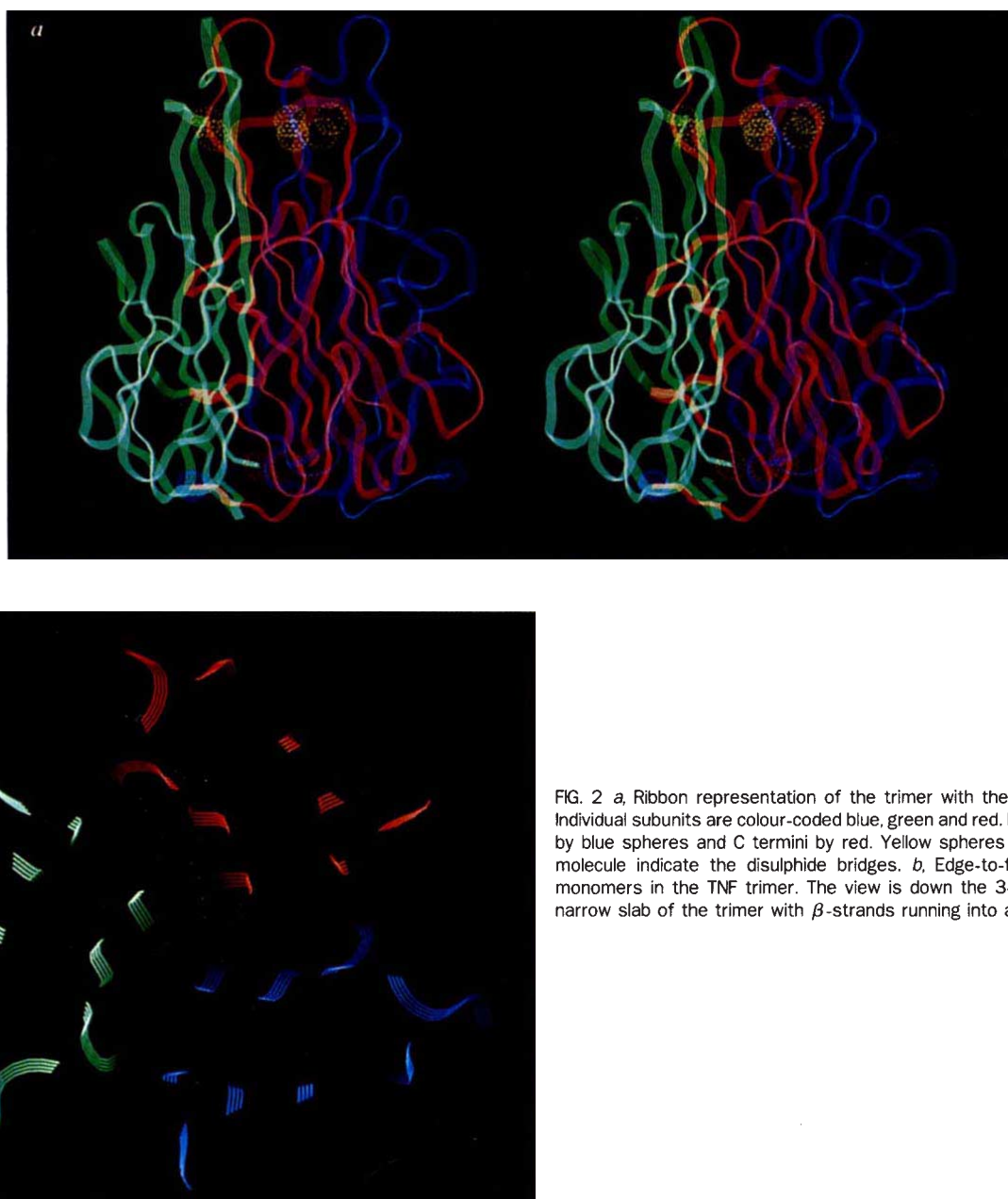


FIG. 2 a, Ribbon representation of the trimer with the 3-fold axis vertical. Individual subunits are colour-coded blue, green and red. N termini are marked by blue spheres and C termini by red. Yellow spheres near the top of the molecule indicate the disulphide bridges. b, Edge-to-face packing of the monomers in the TNF trimer. The view is down the 3-fold axis showing a narrow slab of the trimer with β -strands running into and out of the page.

out using SHP²¹, a program which allows automatic definition of the 'equivalent' residues based on superposition of C α positions.

Foot and mouth disease virus (FMDV) provides an example of the degree of structural homology found between the capsid proteins VP1, VP2 and VP3 of a picornavirus²². Typically 60–70% of the residues are matched with r.m.s. deviations of up to 3.2 Å. The immunoglobulin domain is also an antiparallel β -sandwich but with a totally different connectivity; a comparison of this motif with the jelly-roll fold of FMDV VP3 yields significantly lower values with only about 30% of the VP3 residues matched with r.m.s. deviations of 3.7–4.0 Å. These two results provide useful benchmarks when considering the data for TNF. For a non-jelly-roll β -barrel structure the number of equivalenced TNF residues varies from about 40 to 50% with r.m.s. deviations of between 3.5–4.0 Å. Concanavalin A²³ is an intermediate structure, a considerable portion of this more elaborate molecule can be matched to the viral fold²⁴. This is reflected in the comparison with TNF, for which 64% of the residues of TNF can be matched but with a large r.m.s. deviation of 4.04 Å. The number of equivalenced TNF residues is consistently in excess of 60% with r.m.s. values frequently below 3.5 Å when matched against structures with the jelly-roll motif. Several such comparisons are particularly noteworthy. The structural similarity between TNF and the jelly-roll domain of influenza haemagglutinin (HA)²⁵ is as close as that between HA and FMDV VP3. In the case of two viral capsids, tomato bushy stunt virus (TBSV)²⁶ and adenovirus hexon²⁷, a pair of jelly-roll structures

have been found on the same polypeptide chain, presumably as a result of gene duplication. In both cases, TNF is at least as similar to one jelly roll as the two jelly rolls are to each other. The most striking similarity, however, occurs between TNF and satellite tobacco necrosis virus (STNV)²⁸, a very simple $T=1$ plant virus. 71% of TNF residues can be equivalenced to residues in STNV with only 3.3 Å r.m.s. deviation (Fig. 3b). Thus TNF is structurally as similar to STNV as this protein is to other viral coat proteins.

The structural similarity between the capsid proteins of the small spherical plant viruses, and subsequently the picornaviruses, has been taken to indicate a common evolutionary precursor^{29,30}. The structural similarity described here points to the divergent evolution of TNF and the viral proteins from a common ancestral jelly-roll protein. The identification of additional common characters adds weight to this hypothesis. First, the solvent-accessible outside face in the TNF trimer and in the viral capsid are equivalent. Second, the packing of jelly rolls in the capsid of adenovirus is reminiscent of the TNF trimer. The adenovirus hexon²⁷ contains two jelly-roll domains (P1 and P2) which pack about a quasi 6-fold axis in the hexon. The adenovirus capsid is constructed out of these hexagonal plates such that copies of P1 or P2 from three plates lock into a 3-fold arrangement about each corner of the hexon. Unlike the case in picornaviruses, the longest axis of the β -barrel is roughly normal to the adenovirus surface; hence these 3-fold clusters of P1 or P2 and the TNF trimer have a common general mode of packing. The versatility of the basic jelly-roll motif in fulfilling

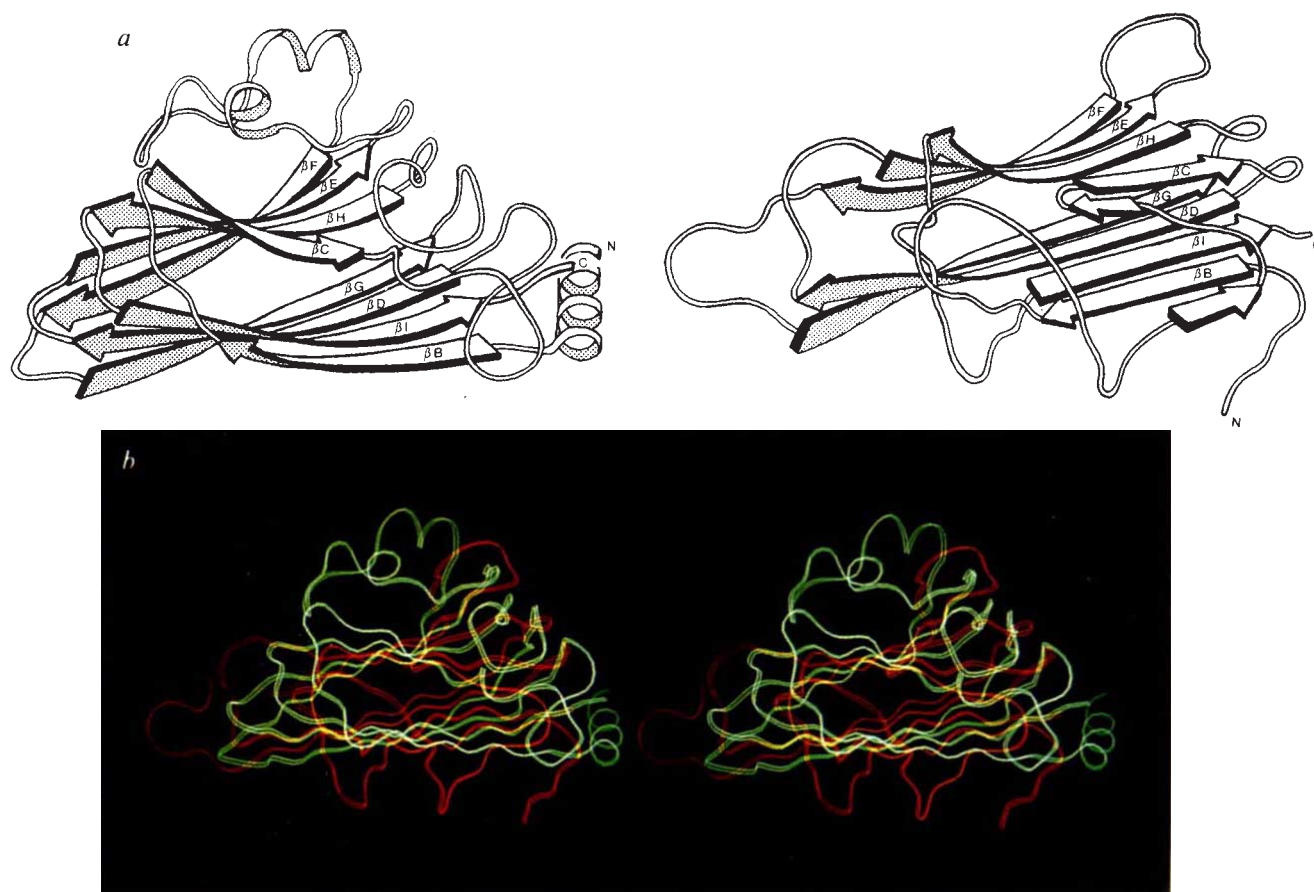


FIG. 3 *a*, Diagrammatic sketch of the polypeptide fold for STNV (top) and TNF (bottom). The β -strands are shown as thick arrows in the amino-to-carboxy direction and are labelled according to the viral convention. An insertion in TNF results in severe truncation of the N-terminal half of β -strand C and addition of short, extra β -strands to the outer edges of both upper

and lower sheets. The TNF trimer 3-fold axis would be horizontal in this orientation. *b*, Superposition of ribbon representations for STNV (green) and TNF (red). The upper β -sheet forms the solvent-accessible surface in both cases.

TABLE 1 Structure determination statistics

Data set	Resolution (Å)	Reflections unique (% complete)	Data collection and phasing		FM (all data, 20–6 Å)	(FHA)/(ETOT) (for minimum FM cutoff)	Wang <i>R</i> -factor	
			<i>R</i> merge† (all data)	Average isomorphous difference			6 Å	4 Å
Native (film)*	2.9	24,385 (76%)	0.125	—				
Native (film)	3.4	16,031 (78%)	0.075					
Native (XEN)	5.0	5,582 (85%)	0.056					
Hg(CH ₃ CO ₂) ₂ (XEN; 5 Å; 3.5 Å)	3.5	14,779 (80%)	0.073	19.11%	0.40	1.7 (0.0)	0.24	0.20
¹ K ₂ Pt(CN) ₄ (XEN, film)	3.4	11,944 (58%)	0.080	17.43%	0.34	2.2 (0.5)	0.26	
K ₂ Hgl ₄ (XEN)	5.0	5,977 (91%)	0.095	27.46%	0.34	2.4 (0.5)	0.25	
Averaging								
			Averaging <i>R</i> -factor	Correlation coefficient	Real space <i>R</i> -factor		TR1	TR2
TR1 and TR2 averaging (2.9 Å)			0.28	0.77			0.27	0.43
TR1 averaging (2.8 Å)			0.19	0.89			0.26	—

Film data were collected on 2° oscillation photographs (3.4 Å resolution) or 1° oscillation photographs (2.9 Å resolution), processed and scaled using standard programs²¹. The data sets collected on the Xenonics area detector (XEN) were taken as 0.2° oscillation frames and processed using the XENGEN program package (A. Howard, XENGEN manual). About 20 heavy-atom compounds were screened for isomorphous derivatives, complete data sets were collected for 15 of these, of which 3 yielded usable data. FHA, heavy-atom structure factor; ETOT, residual lack of closure; FM, figure of merit. Phasing statistics were obtained before any phase modification by solvent flattening. Wang *R*-factor, the crystallographic *R*-factor between observed structure factor amplitudes and those calculated from the solvent flattened map ($R = \sum (|F_o| - |F_c|) / \sum |F_o|$). Averaging *R*-factor, the crystallographic *R*-factor between observed structure factor amplitudes and structure factor amplitudes calculated from the averaged and solvent flattened map. Real space *R*-factor, the *R*-factor between non-crystallographically related electron density. Correlation coefficient, $C = \sum ((F_n) - |F_o|)(\langle F_n \rangle - |F_c|) / [\sum ((F_n) - |F_o|)^2 (\langle F_n \rangle - |F_c|)^2]^{1/2}$, where $|F_o|$ and $|F_c|$ are observed and calculated structure factor amplitudes placed on the same relative scale for each resolution shell, and $\langle F_n \rangle$ is the mean observed amplitude in that shell. TR1, first trimer. TR2, second trimer.

* Including partials. † 10% contribution from Enraf Nonius FAST data. ‡ R merge = $\sum_j \sum_n |I_{h,j} - \langle I_n \rangle| / \sum_j \sum_n I_{h,j}$.

very different packing criteria has been commented on previously^{24,27}.

Conclusions

The structures of three cytokines have so far been established: interleukin-2 (ref. 31), interleukin-1β (ref. 32), and now TNF. Each has a different structural motif. Thus the overlapping biological roles of these molecules are not reflected in their overall structures.

TNF is the first non-viral protein to contain the classic jelly-roll motif. It is a compact molecule shorn of most of the insertions which decorate the jelly roll in the typical viral protein. The basic antiparallel β-sandwich of the jelly roll provides a very stable core arrangement of secondary structure from which connecting loops can provide a myriad of structural variations, a similar principle to that used by the topologically distinct immunoglobulin superfamily. One reason for TNF's rather

austere jelly roll could be that it has no need of great variability as it is not seeking to evade host surveillance. Similarly the STNV coat protein is free from the pressures of escaping an immune system and the more demanding packing requirements associated with the conformational switching of more complex viruses. Hence the greatest similarity occurs between TNF and the coat protein of the simplest of the spherical plant viruses.

If one accepts the case for divergent evolution then our observation accords with the hypothesis of Rossmann *et al.*³⁰ that the viral jelly roll originated from a non-viral receptor binding protein. This homology may in the future provide insight into the functional relationships of these diverse proteins. Our present knowledge suggests that a crucial event in the origins of at least a major class of viruses was the acquisition of the ability to assemble a stable protein shell from subunits based on the jelly-roll motif. It may well be that with the structure of TNF we can start to piece together these origins. □

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- Old, L. *J. Nature* **326**, 330–331 (1987).
- Carswell, E. A. *et al. Proc. natn. Acad. Sci. U.S.A.* **72**, 3666–3670 (1975).
- Gifford, G. E. & Flick, D. A. in 'Tumour Necrosis Factor and Related Cytotoxins' (Ciba Foundation Symposium 131) 3–20 (Wiley, Chichester, 1987).
- Beutler, B. & Cerami, A. *Rev. Biochem.* **57**, 505–518 (1988).
- Mestan, J. *et al. Nature* **323**, 816–819 (1986).
- Wong, G. H. W. & Goeddel, D. V. *Nature* **323**, 819–822 (1986).
- Playfair, J. H. L. & Taverne, J. in 'Tumour Necrosis Factor and Related Cytotoxins' (Ciba Foundation Symposium 131) 192–205 (Wiley, Chichester, 1987).
- Wollheim, F. A., Heinigard, D., Palladino, M., Saxne, T. & Tala, N. *Arthritis Rheum.* **30**, 129 (1987).
- Saklatvala, J. *Nature* **322**, 547–549 (1986).
- Cerami, A. & Beutler, B. *Nature* **320**, 584–588 (1986).
- Smith, R. A. & Baglioni, C. *J. Biol. Chem.* **262**, 6951–6954 (1987).
- Scheurich, P., Ucer, U., Kronke, M. & Pfizenmaier, K. *Int. J. Cancer* **38**, 127–133 (1986).
- Israel, S., Hahn, T., Holtmann, H. & Wallach, D. *Immun. Lett.* **12**, 217–224 (1986).
- Creasey, A. A., Yamamoto, R. & Vitt, C. R. *Proc. natn. Acad. Sci. U.S.A.* **84**, 3293–3297 (1987).
- Yoshie, O., Tada, K. & Ishida, N. *J. Biochem.* **100**, 531–541 (1986).
- Vitt, C. R., Yamamoto, R. & Creasey, A. A. *Fedn Proc.* **46**, 2117 (1987).
- Smith, R. A., Kirstein, M., Fiers, W. & Baglioni, C. *J. Biol. Chem.* **261**, 14871–14874 (1986).

- Wang, B. C. *Meth. Enzym.* **115**, 90–111 (1985).
- Bricogne, G. *Acta Crystallogr.* **A32**, 832–847 (1976).
- Brünger, A. T., Kuriyan, J. & Karplus, M. *Science* **235**, 458–460 (1987).
- Stuart, D. I., Levine, M., Muirhead, H. & Stammers, D. K. *J. molec. Biol.* **134**, 109–142 (1979).
- Acharya, R. *et al. Nature* **337**, 709–716 (1989).
- Reeke, G. N., Becker, J. W. & Edelman, G. M. *J. biol. Chem.* **250**, 1525–1547 (1975).
- Argos, P., Tsukihara, T. & Rossmann, M. G. *J. molec. Evol.* **15**, 169–179 (1980).
- Wilson, I. A., Skehel, J. J. & Wiley, D. C. *Nature* **289**, 366–373 (1981).
- Harrison, S. C., Olson, A. J., Schutt, C. E., Winkler, F. K. & Bricogne, G. *Nature* **276**, 368–373 (1978).
- Roberts, M. M., White, J. L., Grütter, M. G. & Burnett, R. M. *Science* **232**, 1148–1151 (1986).
- Liljas, *et al. J. molec. Biol.* **159**, 93–108 (1982).
- Rossmann, M. G. *et al. J. molec. Biol.* **165**, 711–736 (1983).
- Rossmann, M. G. *et al. Nature* **317**, 145–153 (1985).
- Brandhuber, B. J., Boone, T., Kenney, W. C. & McKay, D. B. *Science* **238**, 1707–1709 (1987).
- Priestle, J. P., Schär, H.-P. & Grütter, M. G. *EMBO J.* **7**, 339–343 (1988).

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A laminin-like adhesive protein concentrated in the synaptic cleft of the neuromuscular junction

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A striking example of topographic specificity in synapse formation is the preferential reinnervation of original synaptic sites on denervated muscle fibres by regenerating motor axons. This specificity is mediated by the basal lamina of the synaptic cleft. A glycoprotein, s-laminin, has now been identified that is selectively associated with synaptic basal lamina and is recognized by motoneurons. Molecular cloning reveals that s-laminin is a novel homologue of laminin, a potent promoter of neurite outgrowth.

IN most vertebrate skeletal muscles, each muscle fibre is innervated at a single site by a single motor axon. Injuring the motor nerve leaves the muscle denervated, but axons can regenerate to form new neuromuscular junctions that resemble their normal counterparts in structure and function. A striking and

consistent feature of reinnervation is that it is topographically specific: axons preferentially form new junctions at original synaptic sites¹⁻³. The fidelity of this process—in some cases more than 95% of the regenerated synapses form on the ~0.1% of the muscle fibre surface that was originally synaptic³—indicates that there are factors closely associated with original synaptic sites that axons recognize.

We have shown previously that some of these factors are associated with the basal lamina (BL) that ensheathes each muscle fibre and passes through the synaptic cleft at the neuromuscular junction^{3,4}. Subsequently, we^{5,6} and others^{7,8} generated antibodies that selectively stained synaptic sites on muscle fibre BL, revealing molecular correlates of the functional specialization demonstrable during reinnervation. Here, we identify one such synaptic antigen as a novel homologue of laminin, a glycoprotein of the BL already known to be adhesive for neurons⁹. Because the distribution of this laminin-like protein is restricted to a subset of BLs, including synaptic BL, we call it s-laminin. Its location, bioactivity and resemblance to laminin suggest that s-laminin is involved in the formation or stabilization of synapses.

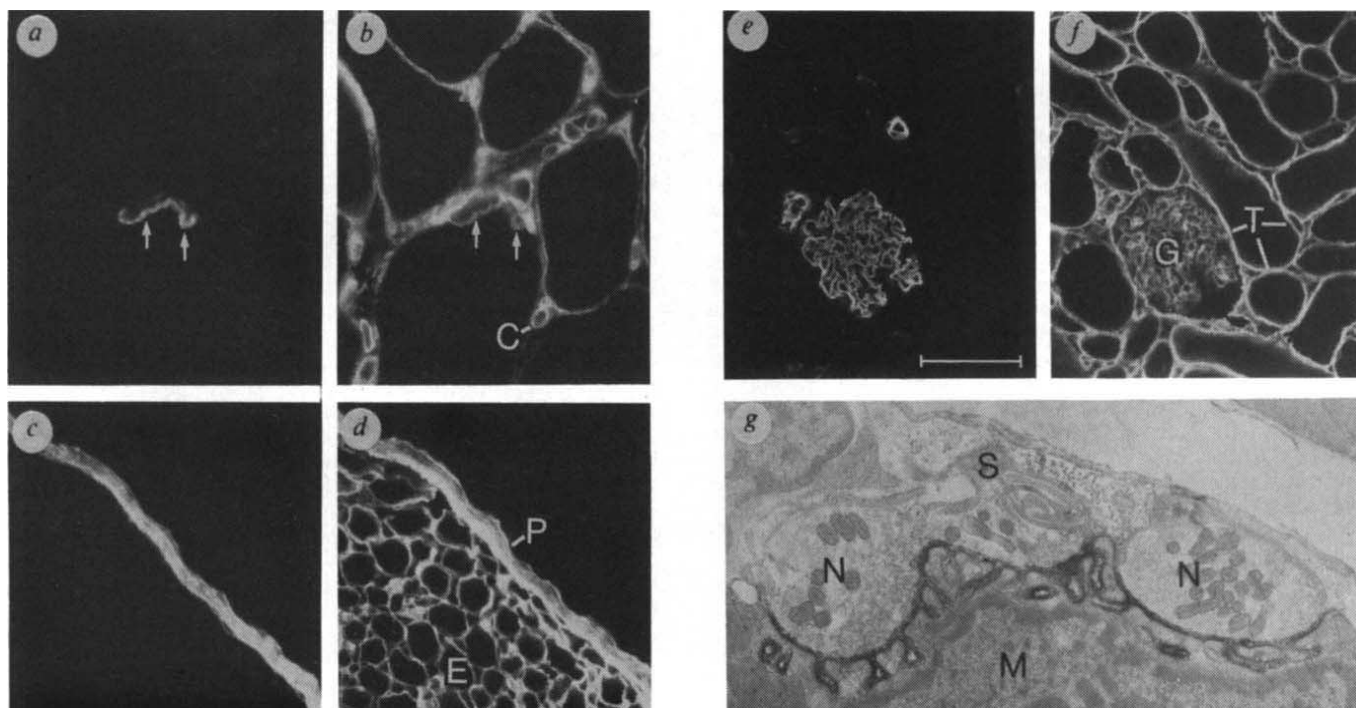


FIG. 1 S-laminin is restricted to a subset of laminin-rich BLs. Cryostat sections of rat skeletal muscle (*a, b*), sciatic nerve (*c, d*) or kidney (*e, f*) were doubly stained with anti-s-laminin mAb D7 plus fluorescein goat anti-mouse IgG (*a, c, e*) and with anti-laminin serum plus rhodamine goat anti-rabbit IgG (*b, d, f*), as described⁴⁴. S-laminin is concentrated in synaptic (arrows) but not extrasynaptic or capillary (C) BL in muscle (*a, b*), in perineurial (P) but not endoneurial (E) BL in nerve (*c, d*) and in glomerular (G) but not tubular (T) BL in kidney (*e, f*). *g*, Electron micrograph of a neuromuscular junction from a

muscle that had been stained with mAb C1 plus horseradish peroxidase second antibody⁴⁴. Reaction product is concentrated in synaptic BL between nerve terminals (N) and muscle fibre (M); extrasynaptic and Schwann cell (S) BLs are not detectably stained. In experiments not shown here, we used methods described in ref. 5 to show that mAbs to s-laminin stained the BL in the synaptic cleft, rather than the pre- or postsynaptic membranes. Scale bar in *e*, 50 μ m for *a-d* and 100 μ m for *e, f*. Scale bar in *g*, 1 μ m.

Restricted distribution of s-laminin

S-laminin was initially defined by an antiserum (JS-1) to an extract of lens capsule BL, which stained synaptic BL⁵. Because the antiserum was not monospecific, we generated four monoclonal antibodies (mAbs; C1, C4, D5 and D7; see refs 6, 10) that recognized JS-1-related antigens: each stained synaptic BL (Fig. 1a, g) and staining by each was blocked by preincubation of the sections with JS-1 (data not shown). All four mAbs recognized the same polypeptide, s-laminin (see below), but they differed in species cross-reactivity and in reactivity with denatured antigen, indicating that each mAb recognizes a distinct epitope. The selective staining of synaptic BL by these mAbs is therefore likely to reflect restricted distribution of s-laminin rather than differential accessibility of a uniformly distributed determinant.

Although s-laminin is concentrated at synaptic sites in muscle, it is abundant in some non-muscle BLs. In peripheral nerve, all

four mAbs to s-laminin stained perineurial BL more intensely than endoneurial BL (Fig. 1c). In kidney, these mAbs stained glomerular BL more intensely than tubular BL (Fig. 1e). Within muscle, arterial BL was stained more strongly for s-laminin than venous or capillary BL. All BLs were strongly and similarly stained by antibodies to laminin (Fig. 1b, d, f) and to a heparan sulphate proteoglycan¹¹. Thus, s-laminin is restricted to a subset of BLs both in muscle and elsewhere. Interestingly, in the tissues examined so far, BLs rich in s-laminin are apposed on both sides by cellular membranes (for example, nerve and muscle at the synapse, endothelium and podocyte in the glomerulus), whereas BLs poor in s-laminin separate cells from interstitial connective tissue¹².

Identification of s-laminin

Because glomerular BL has been characterized in detail¹³, we used kidney to identify the antigen(s) recognized by mAbs C1, C4, D5 and D7. BL-rich fractions were analysed by immunoblotting. When samples were not reduced, the antibodies recognized only material with a relative molecular mass (M_r) of more than 10⁶ (1,000K). Under reducing conditions, however, C4, D5 and D7 all stained a band of apparent M_r ~190K (Fig. 2a). C1 did not stain immunoblots, but C1-agarose precipitated C4-reactive material of about 190K. Immunoblots of two-dimensional gels (data not shown) and chromatofocusing chromatography (Fig. 2b) demonstrated that these mAbs recognized the same protein; it has an isoelectric point of ~6.5. Thus, a ~190K protein is identified as a subunit of s-laminin. In addition, C4 and D7 weakly stained a band of M_r ~400K; we have not studied the relationship of the ~400K material to the ~190K s-laminin.

The insolubility of s-laminin confirmed its association with the extracellular matrix. Little immunoreactive material was released upon incubation of s-laminin-rich fractions with high salt solutions, non-ionic detergents, reducing agents or strongly chaotropic agents. Extraction with a chaotrope (guanidine-HCl) plus a reducing agent (dithiothreitol) did, however, release most of the s-laminin detectable by immunoblotting. Collagenase treatment did not affect the M_r of extracted s-laminin. Solubil-

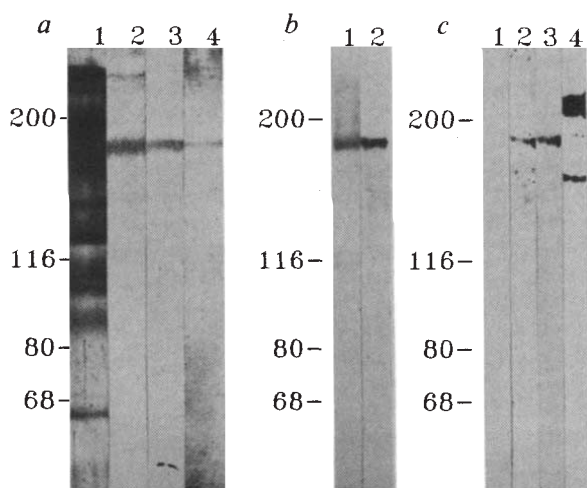


FIG. 2 Identification of s-laminin by immunoblotting. a, A crude extract of renal cortex was analysed by gel electrophoresis and either silver-stained (lane 1) or transferred to nitrocellulose and probed with mAbs C4 (lane 2), D5 (lane 3), or D7 (lane 4). Positions of relative molecular mass standards are indicated in thousands; the M_r of s-laminin is ~190K. b, Peak fraction from chromatofocusing column, silver-stained (lane 1) or immunoblotted with mAb C4 (lane 2); compare with lanes 1 and 2 in a. c, Renal extract, prepared as in a, and immunoblotted with normal serum (lane 1), a polyclonal antiserum to a fusion protein encoded by s-laminin cDNA RK65-6 (lane 2), mAb D5 (lane 3), or a polyclonal antiserum to laminin (lane 4).

METHODS. Sample preparation and electrophoresis were performed as described⁴⁵, using 5% polyacrylamide gels. Electrophoretic transfers to nitrocellulose (S&S BA85) was as described⁴⁶; transfers were immunostained with 1:1,000 dilutions of anti-s-laminin antibodies or rabbit anti-laminin serum (Polysciences) and alkaline phosphatase-second antibody (1:1,500; Boehringer), according to the manufacturer's instructions. Transferred relative molecular mass markers were stained with 0.2% Ponceau S (Eastman Kodak) in 3% trichloroacetic acid (Aldrich) and 3% sulphosalicylic acid (Sigma). To purify s-laminin, homogenates of renal cortex were successively treated with 2M MgCl₂, 3% NP-40 and 6M guanidine-HCl to remove soluble, membrane-bound and chaotrope-soluble components. S-laminin was then extracted from the residue with 6M guanidine-HCl plus 10 mM dithiothreitol, resulting in a purification of ~100-fold. After dialysis, chromatofocusing chromatography was performed on PBE 94 (Pharmacia), following the manufacturer's instructions. Briefly, BL extract was dissolved in 7 M urea (ICN-Schwarz Mann) plus 5% β -mercaptoethanol, 2% NP-40, 25 mM imidazole pH 7.4, applied to a 1 cm by 10 cm column equilibrated with the same solution, and eluted with 7 M urea, 5% β -mercaptoethanol, 2% NP-40, 12.5% polybuffer 74 (Pharmacia) pH 4.0. Fractions were assayed for s-laminin by immunoblotting and for protein content by dye binding (Bio Rad). S-laminin was eluted at a pH of ~6.5. To prepare antisera, RK65-6 was recloned into the pET vector⁴⁰, *Escherichia coli* were transformed and the protein was purified from a cell lysate by gel electrophoresis; gel slices containing 0.5 mg of fusion protein were homogenized in RIBI adjuvant and injected into rabbits.

FIG. 3 a, Alignment of cDNA clones which encode s-laminin. The location of clones isolated from rat kidney (RK) and muscle (RM) λ gt11 libraries are shown on a schematic sequence. Loops represent introns found in RK65-3. Insert indicates immunoreactivities of fusion proteins generated from cDNAs with mAbs described in the text. b, Nucleotide and deduced amino-acid sequence of s-laminin. Predicted domains (see text), and their respective residues, are VI (1-248), V (249-520), IV (521-749), III (750-1,158), II (1,159-1,377), α (1,378-1,410) and I (1,411-1,766). Residues identical to those in mouse laminin B1¹⁸ are underlined. The seven potential N-glycosylation sites (laminin B1 contains 14; see ref. 18) are in bold face. A predicted signal peptide of 35 amino acids (residues -35 to -1) is similar to that of laminin B2. If the signal peptidase cleavage site is located after the sequence Thr-Leu-Ala, in agreement with the '-3, -1' rule⁴⁷, s-laminin begins with glutamine, as do human and mouse laminin B1, human laminin B2 and mouse laminin A (refs 18, 19, 21, 23). A polyadenylation site (underlined) precedes the poly(A) tail by 12 bp.

METHODS. RK27 and RK36 were isolated from a kidney expression library (Clontech) using anti-s-laminin antibodies at 1:1,500 dilutions and 1:2,000 dilutions of an alkaline phosphatase-goat anti-mouse IgG, using published procedures⁴⁸. RK65-3 and RK65-6 were isolated from the same library by hybridization at 42°C with a ³²P-labelled fragment of RK27, as previously described³⁶; washes were at 65°C in 0.1 × SSC containing 0.1% SDS. RM12 and RM15 were isolated from a λ gt11 library constructed from rat muscle poly(A)⁺ RNA as previously described⁴⁹, using a ³²P-labelled fragment of RK65-3 (with washes as above) and a ³²P-labelled octadecanucleotide near the 5' end of RK65-3 (with washes at 58°C in 6 × SSC containing 0.1% SDS). The nucleotide sequence was determined after subcloning into Bluescribe (Stratagene), with a modification of the dideoxy chain termination method⁵⁰, using Sequenase (USB), oligonucleotide sequencing primers (synthesized at the Protein Chemistry Laboratory of Washington University) and α -[³⁵S]-dATP (NEN) as the label. All clones shown were sequenced on both strands.

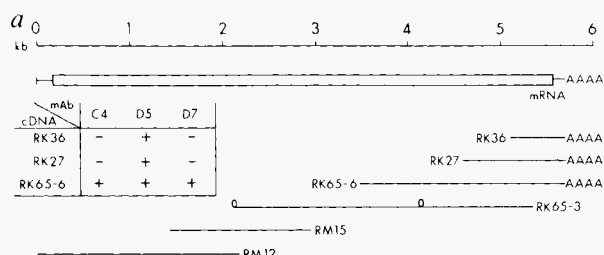
ized s-laminin bound to concanavalin A-agarose and wheat-germ agglutinin-agarose and could then be eluted by appropriate haptens. These data indicate that s-laminin is a non-collagenous glycoprotein tightly attached to BL by a combination of hydrophobic and disulphide bonds.

Neuronal adhesion to s-laminin

As a first step in testing the hypothesis that regenerating axons interact with s-laminin at synaptic sites, we tried to determine whether neurons adhere to this protein. Neurons were incubated

in s-laminin-coated tissue culture dishes for 2 h at 37°C; unbound neurons were then rinsed away and adherent neurons counted. Chick ciliary neurons were chosen for these assays because they innervate striated muscle *in vivo*¹⁴ and recognize synaptic sites on adult muscle fibres *in vitro*¹⁵.

Neurons adhered comparably well to laminin and to a preparation of s-laminin that had been purified ~2,000-fold from renal cortex by selective extraction and chromatofocusing (Table 1, series A). Several other proteins were not adhesive in this assay. The s-laminin preparation contained no detectable laminin or



a

0 1 2 3 4 5 6

kb

1

ACTGAGTCT CTGCTGGAC CCGTGGACAG ACTGAGGCG TGGAGGAA CCAGCCCAT ACCACACGG

69 ATG GAG TGG GGC TCA GGA AAA CCA GCG AGC GGC AGG CAG GGA CAG CTT GTG CCA TGC GAA

129 CTT CCG TTG GGC CTA CTT CTA AGT GTG CTG GCT GGC CCA TGC GGC CAA GTG CCA TGC TGC

179 Leu Arg Leu Ser Gly Lys Leu Ser Val Leu Ala Ala Thr Leu Ala Gln Val Pro Ser Leu

189 GAT GTA CTT GGC TGT TCT CCA GGA ACC TCT TAT CCA GAT GAT GAT GAT GAT GAT GAT GAT

249 CCG GCA CAG CAG CCA GGC TCC AGC TGT GGC TGC CAT AGC CTT GAA CCG TAC TGT

26 Arg Ala Asp Arg Leu Thr Ala Ser Ser Thr Cys Gly His Ser Pro Gln Pro Tyr Cys

309 TCT CTT ACT CAG CAG GAT GAA AAG AAG CCG TGC TGC TGC TGC TGC TGC TGC TGC

46 TCT CTT CCA GAG AAC CAA ACT GAT CAG CCG AGC TGC TGC TGC TGC TGC TGC TGC

66 Ser Ala Arg Asp Asn Pro Asn Ser His Arg Ile Gln Asn Val Val Thr Ser Phe Ala Pro

129 CAA CCG AGC CCG TGG TGG CAG TGC GAG AAC GGT GTT CCG ATG GTC ACC ATT CAA CTC

86 Gln Arg Arg Thr Ala Trp Trp Gln Ser Gln Asn Gly Val Pro Met Val Thr Ile Gln Leu

489 CAG CTG GAA GCT GAT TTT CTT TCC CAG CAG CTT CTT CTT CTT CTT CTT CTT CTT CTT

106 Asp Leu Gln Ala Glu Phe His Phe Thr His Leu Ile Met Thr Phe Lys Thr Phe Arg Pro

549 GCG GCG ATG CTG GTC GAG CCG TCC CCA GAG TTC GGC CCG AGC TGC GGT GTC TAC GGA TAT

126 Ala Ala Met Leu Val Glu Arg Ser Ala Asp Phe Gly Arg Thr Trp Arg Val Tyr Gln Val

609 TTT TCC TAC GAG TGC GGC GGT GAT CCA GGA ATC CCG CTG CCG CCG CCG CCG CCG CCG

146 Phe Ser Tyr Asp Cys Gly Ala Asp Phe Gly Ile Pro Leu Ala Pro Phe Arg Arg Trp

669 GAT GAT GTA GTG TGT CCG TCC GGC TAC TCA GAA ATT GAG CCA TCT GAA GGC GAG GTC

126 Asp Asp Val Val Glu Ser Arg Tyr Ser Arg Ile Gln Pro Leu Ala Pro Phe Arg Arg Trp

729 ATC TAT CTT GTG CTT GAT CTT ATT CTT ATT CTT ATT CTT ATT CTT ATT CTT ATT CTT

186 Ile Tyr Arg Val Leu Asp Pro Ala Ile Pro Ile Pro Asp Pro Tyr Ser Arg Ile Gln

789 AAC CTG TGC AAG ATT ACC AAC CTC CCG GAT CAG CTT CCG CTT CAG CCA TGC GAG CAG

206 Asn Leu Leu Lys Ile Thr Asn Leu Arg Val Asn Leu Thr Arg Leu His Thr Leu Gly Asp

849 AAC TTG CTT CAG CCA CCG CCG GAT ATT CCG GAA AAA TAT TAT TAT TAT TAT TAT TAT

226 Asn Leu Leu Asp Pro Arg Arg Arg Ile Ala Ser Gln Cys Asn Gly His Asn Thr Arg

909 GTC ATC CCG CCG AAC TCC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC

246 Val Ile Arg Gly Asn Cys Phe Cys Tyr Gly His Ala Cys Ile Cys Lys His Asn Thr Arg

969 GCA CCG GCG CAT GCT GAG GCG ATG GTA CTT GCG GCG TGT ATT TGC AAG CAA AAT ACT CCG

266 Thr Pro Ala His Ala Glu Gly Met Val His Gly Ala Cys Ile Cys Lys His Asn Thr Arg

1029 GCG CTC AAC TGT GAG CAG TGT CAG ATT TTT TAT CAG CAG CTT CCG TCG CTT CCG TCG

286 Gln Leu Asn Cys Gln Gln Cys Gln Asn Phe Tyr Phe Thr Pro Trp His Pro Trp His

1089 GAT GCG CAT ACT CAG CCG TGT CCG AAC TGT GAG TCC AAC GCG CAT GAT AGC TCC CAG

306 Thr Gly His Thr His Ala Glu Ser Arg Lys Cys Asn Gly His Ser His Ser His Thr Arg

1149 TTT CAG ATT CTT CTT TAC TCA GCA TCT GCA AAG GTA AGT GGA GGT GTC TGC GAT GCG TGT

326 Phe Asp Met Ala Val Thr Leu Ser Gly Asn Val Ser Gly Val Val Cys Asp Gly Cys

1209 CAG CAG AAC CCG GGT GCG GCG CAG TGC CAG CTT CCG CCG CCG CCG CCG CCG CCG

1306 Gln His Asn Thr His Gln Leu Cys Gln Leu Cys Arg Pro Phe Phe Tyr Arg Asp Pro

1269 ACC AAG CAG ATG CCG GAT CCA CCG TCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

166 Thr Lys Asp Thr Lys Asp Pro Ala Ala Cys Arg Pro Cys Asp Cys Asp Pro Met Gly Ser

1379 CAA CAG GGT CCG CCG TGT CCG TGT CCG TGT CCG TGT CCG TGT CCG TGT CCG TGT

186 Gln Asp Gly Cys Arg Cys Asp Ser His Asp Asp Pro Val Leu Gly Leu Val Ser Cys Cys

1439 TCT CCG TCC AAA GAA CAG CTT GTT CCG ACT CCG TCC CAG TCC CCG CCG CCG TTT

186 Cys Arg Cys Lys Gln His Val Val Gly Thr Arg Cys Gln Gln Cys Asp Gly Phe

1409 GCA CTT ACT CCG GCG AAC CCA GCA TGT CAG CCG TGT CAG TGT CAG TGT CAG TGT

426 Thr Lys Ser Ala Ser Asn Arg Arg Gly Cys Gln Arg Cys Asn Ser Arg Arg Gly

1549 CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

446 Val Pro Gly Gly Thr Pro Cys Asp Ser Ser Gly Thr Cys Phe Cys Lys Arg Leu Val

1569 ACT GCA GCG CCG TGT CAG CCG TGT CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

466 Thr Gly Asp Gly Cys Asp Arg Cys Leu Pro Gly His Trp Gly Leu Ser His Asp Leu Val

1629 GCG TCG CCG CCG TGT CAG TGT GAT GTG CCG GGT GCG TGT GAT CCG TGT GAT GAA GCG

486 Gly Cys Arg Pro Cys Asp Cys Val Gly Gly Ala Leu Asp Pro Gln Cys Asp Glu Ala

1689 ACG GGT CAG TCG CCG TCC CCG CAG ATG ATT GCG GCG CCG TGT GAA CAA CTA CAG CCG

506 Thr Gly Gln Cys Pro Cys Arg Pro His Met Ile Gly Arg Arg Cys Glu Gln His Thr Pro

1749 GCG TAC TTC CCG CTT TTT CTA CAG CTA CTA CCG CAG GGT GAG GGT GCG CCG CCG CCG

526 Gly Tyr Phe Arg Pro Phe Leu His Leu Thr Trp Glu Ala Glu Gly Ala Thr Met

1809 CTT CTT CAG GTG GTA GAA GGT CTT GGT ACC AAC CCA GAG ACT CCG TCC TGT GAT GGT

546 Val Leu Glu Val Val Glu Arg Leu Val Thr Asn Arg Gln Thr Pro Ser Trp Thr Gly

1869 GCG TTT GTG CCA CCG GAA GAT CAG GAA GAT CAG GAG TGT CTT CCG ACC TGT CTT CCG

566 Gly Phe Val Arg Leu Arg Glu Gly Gln Val Thr Ser Leu Val Thr Ser Leu Val Thr

1929 CCG ATG CAG TAT CAG CTT CTA CCG CCG TCG GAG CCG CCG CCG CCG CCG CCG CCG

586 Ala Met Asp Tyr Asp Leu Leu Leu Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

1989 CTG GAA TTG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

606 Leu Glu Leu Val Val Gln Arg Pro Gly Pro Val Ser Ala His Ser Pro Cys Gly His Thr

2049 CCG CCG AGC GAT CAG CCG ATT CAG GGT CTT CAG CCA AAC ACC AGG GTT TTG TGT Phe

626 Thr Pro Arg Asp Asp Arg Ile Gln Gly Met Leu His Pro Asn Thr Arg Val Leu Val Thr

2109 CCG AGA CCG CTT CAG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

646 Thr Arg Pro Val Cys Leu Glu CCG GGT CCG TCC TAC AAG CCG AAG TTT AAA TGT ACT GAG

666 Thr Gly Gly Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

2169 CAA GCG GCA CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

686 Thr Val Leu Glu Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

2229 CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

706 Thr Glu Arg Arg Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

2289 CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

726 Thr Glu Arg Arg Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

2349 AAG ACC CCG CTA TCT CAG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

746 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

2409 AAT CCG CCG TCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

766 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

2469 CAG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

786 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

2529 ACT GGC TAC TAT GGC TTT GGC GGC CCA GGC TGT GAA GGC TGC CAG TGT AGT CCG GAG GGA

786 Thr Gly Tyr Tyr Gly Phe Gly Gly Gly Cys Thr Thr Thr Thr Thr Thr Thr Thr

2589 CCA CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

806 Ala Leu Ser Ser Gly Leu Cys Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

2649 CTT CCG TGT CAG CAG TGT CCA CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

846 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

2709 GTC TCC AAC GGC CCG CCA GAT CAG TGT CAG CCG CCA CCA CCG CCG CCG CCG

866 Val Cys Asn Gly Arg Ala Asp Gly Cys Ala His Thr Thr Thr Thr Thr Thr Thr

2769 GAT TAC ACA GCG GCG GAG CAG TGT GAG AGG TCC ATT GCT GCT TTT CAG CCG CCG

886 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

2829 CTG CCA TAT GCG GCG CAG TCC CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

906 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

2949 CCA GCG TAC ACA GCG CTT CCG TGT CAG GGT TGT GCG CCG CCG CCG CCG CCG

926 Ala Gly Tyr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

3009 AAG CCA GGT GCG AGG TCC CAA CCG TGT CAG TCC AGT GCG AAC ATT CAG CCG

946 Lys Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

3069 GCG CCG TGT GAT CCG CAG CCG GCG CAA TCC TGC GGT TTA CAG CCG CCG CCG

966 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

3129 CTT CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

986 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

3189 TCT ACC TCC AGC CTT CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1006 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

3249 CCG CCA CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1026 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

3309 CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1046 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

3369 CCA CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1066 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

3429 CCG CCG TTT GGT GCG AGG ACC TGT TGT CAG CCG CCG CCG CCG CCG CCG

1086 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

3489 CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1106 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

3549 TCT ACT GGT CAG TGT AGC TCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1126 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

3609 CCG CCG TCG TCG GGT TTT TCT CCG CCG CCG CCG CCG CCG CCG CCG CCG

1146 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

3669 ATG GGT GTG GTA CAG GAG Leu Ala Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

1166 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

3729 TGT CAG CAA GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT

1186 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

3789 TGT CAG CAA GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT

1206 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

3849 CTT GTA GAG CCG ACA GAG GGT CCG CCG CCG CCG CCG CCG CCG CCG CCG

1226 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

3909 CCG TTA GAA GAG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1246 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

3969 ACT GGT CCG GAG ACA GAG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1266 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

4029 CCG GAT ACT CCG AAA CAG CCA AAT TTT TTA GGT CCG CCG CCG CCG CCG

1286 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

4089 ACT CAG TCT ACA GAA GAG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1306 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

4149 GCG AAC TCA CCA GAT ACC CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1326 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

4209 AAC TTA CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1346 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

4269 CAG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1366 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

4329 TGT CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1386 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

4389 CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1406 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

4449 CAG CCA CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG

1426 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

4509 TGT CAG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1446 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

4569 CAG CAG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1466 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

4629 AAT GTC AAG CAG TTT CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1486 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

4689 CAG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1506 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

4749 GAG ATT CCA GAA CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1526 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

4809 GCG GAT GTG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1546 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

4869 GCG GAT GAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG

1566 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

4929 CAG CCA CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1586 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

4989 CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1606 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

5049 GCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1626 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

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1646 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

5169 CCG GAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG

1666 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

5229 GAA CCG AAG CCG GAA GGT GTT CCG CCG CCG CCG CCG CCG CCG CCG CCG

1686 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

5289 CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1706 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

5349 CAA TAT GAA CAG AAT GAA GCG GAA CCG CCG CCG CCG CCG CCG CCG CCG

1726 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

5409 CCG AGG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1746 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

5469 CAG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG CCG

1766 Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

fibronectin (as judged by immunoblotting and silver-staining of SDS gels), but was not homogeneous. These results indicate that neurons adhere to s-laminin, but do not exclude the possibility that contaminants in the s-laminin preparation are involved. Neurons also adhered to s-laminin prepared by hydroxylapatite or mAb D5-agarose chromatography (Table 1, series A) however. Thus, neurons do adhere to s-laminin or to molecules that co-purify with s-laminin by three different methods.

Cloning of s-laminin cDNA

We used molecular cloning to obtain structural information about s-laminin and a source of recombinant protein for further assays of function. A rat kidney cDNA library in the expression vector λ gt11 was screened with seven mAbs to s-laminin (the four described above and three others). From 7×10^6 plaques, we identified two clones that reacted with mAb D5 (RK27 and RK36; Fig. 3a). Using a fragment of RK27 as a probe, clones RK65-3 and RK65-6 were isolated by screening an additional 1.5×10^6 plaques. A 5' fragment of RK65-3 was then used to screen 1.5×10^6 plaques from a rat muscle cDNA library; 19 clones, including RM12 and RM15, were isolated.

Sequence analysis indicates that these cDNAs span a distance of 5.6 kilobases (kb), comprising 68 base pairs (bp) of 5' untranslated region, a single open reading frame of 5,403 bp, a 3' untranslated region of 110 bp and a poly(A) tail (Fig. 3b). Monoclonal antibodies C4, D5 and D7 all bound specifically to a fusion protein encoded by RK65-6 (see Fig. 3a), indicating that the cDNA-encoded protein shares three distinct epitopes with s-laminin. Furthermore, antisera to RK27 and RK65-6 fusion proteins stained synaptic sites in sections (data not shown) and recognized s-laminin on immunoblots (Fig. 2c).

Sequences of cDNAs derived from muscle and kidney are identical in regions of overlap. On northern blots, RK65-6 hybridized to a single mRNA of ~5.7 kb in RNA from kidney and muscle (data not shown). Together, these results demonstrate that we have cloned virtually the entire s-laminin mRNA.

S-laminin mRNA encodes a protein with a predicted M_r of 196,472 that is rich in cysteine, has seven potential *N*-glycosylation sites and includes an *N*-terminal signal sequence of $M_r \approx 3,471$ characteristic of membrane-spanning and secreted proteins (Fig. 3b). These features are consistent with the properties of s-laminin described above. The most striking feature of the s-laminin sequence, however, is its homology to the laminin subunits. Laminin is composed of three subunits, A, B1 and B2, arranged in a cruciform structure¹⁶. On the basis of their predicted secondary structures, the laminin subunits have been divided into several domains. Domains I and II contain heptad repeats thought to form coiled-coil α -helices; III and V contain ~50-residue-long cysteine-rich repeats postulated to form loops; and IV and VI are predicted to be predominantly globular¹⁷⁻²⁴. In the B1 but not the A or B2 subunits, domains I and II are separated by a short cysteine-rich domain, called α ¹⁸. S-laminin contains all seven domains, I-VI plus α ; matrix analysis highlights the repeats and the borders between domains (Fig. 4a). S-laminin has more similarity with the B1 subunit of laminin than with A and B2; all of the subunits are related to each other (Fig. 4b).

Several regions of the laminin B1 molecule have been implicated in functional interactions. The sequence YIGSR (in the one-letter code) in domain III adheres to cells of several types²⁵; s-laminin contains the sequence YTGLR in a corresponding position. A 20-mer from domain IV

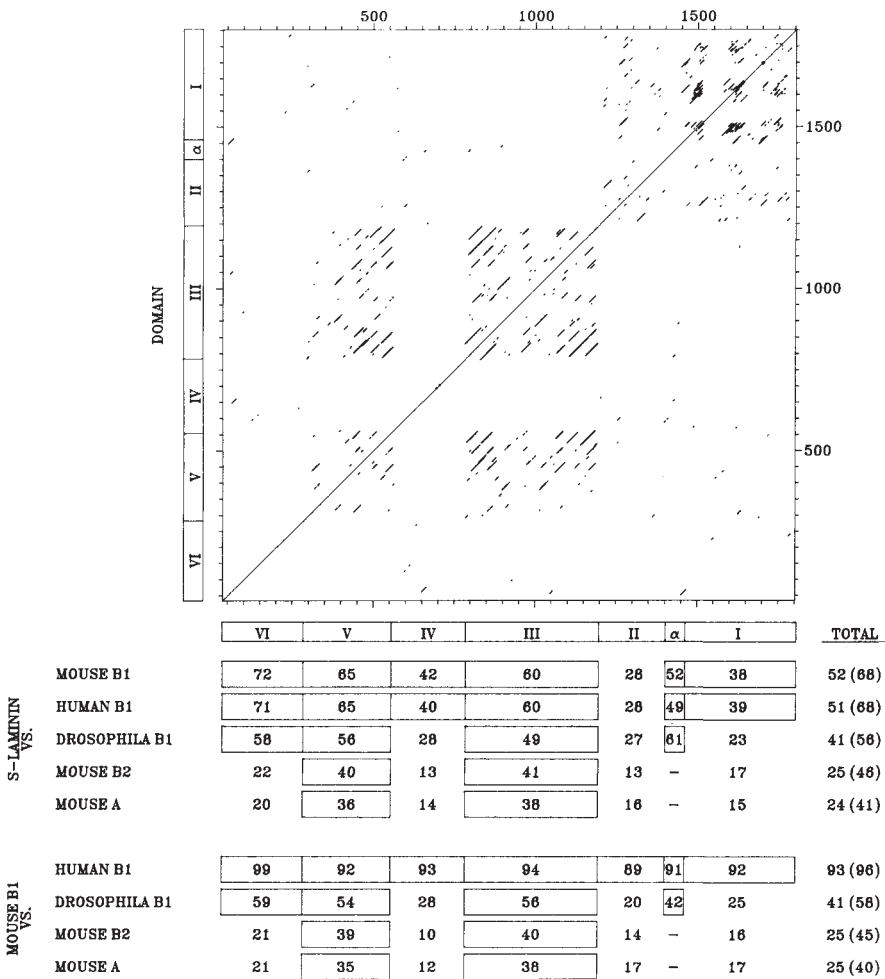


FIG. 4 a, Dot matrix plot comparing predicted amino-acid sequence of s-laminin to itself, to demonstrate internal repeats. Dots indicate positions at which more than 16 amino acids in a stretch of 30 are identical. Numbering of residues is from Fig. 3. Borders between domains are indicated along the axes. Multiple repeats in domains I-III and V are evident. b, Homologies (percentage identity) between amino-acid sequences of s-laminin and the subunits of laminin. Values of $\geq 35\%$ are boxed. Sequences and their divisions into domains are from refs 17-24. Homologies were computed using the UWGCG GAP program with gap penalty = 0.3. Gap weight = 1 for *Drosophila* α and 5 for all other comparisons. For laminin A, domain G was omitted and scores for domains III a and b were averaged, as were scores for IV a and b. Values in parentheses under "total" are percentage similarity, in which evolutionarily conservative substitutions are given some weight. Note that s-laminin and laminin B1 are equally homologous to the *Drosophila* sequence that has been termed B1.

TABLE 1 Adhesion of ciliary neurons to s-laminin

Substrate		Neurons per field
Series A		
Laminin		21 ± 1
s-laminin	Chromatofocusing peak	15 ± 1
	Hydroxylapatite peak	24 ± 2
	D5-agarose eluate	20 ± 1
Bovine serum albumin		3 ± 1
Series B		
Laminin	+mAb C14	25 ± 2
	+mAbs C4 + D5	22 ± 2
		23 ± 3
Recombinant s-laminin (pET-RK65-6)		29 ± 3
	+mAb C14	25 ± 6
	+mAbs C4 + D5	3 ± 0

Laminin (Collaborative Research; 0.1 mg ml⁻¹), purified s-laminin (0.01 mg ml⁻¹), recombinant s-laminin (0.01 mg ml⁻¹), or bovine serum albumin (BSA) (Sigma; 30 mg ml⁻¹) were applied to nitrocellulose-coated tissue culture dishes as described⁴³; free sites on the nitrocellulose were then blocked with 30 mg ml⁻¹ BSA for 2 h. Neurons dissociated from embryonic-day-10 chick ciliary ganglia¹⁵ were suspended in medium (DMEM with 1 mg ml⁻¹ BSA and 10 mM HEPES, pH 7.3) and added to the dishes. After 2 h of incubation at 37 °C, plates were rinsed twice with medium, adherent cells were fixed with 2% formaldehyde and 2% glutaraldehyde and neurons were counted. Wells containing antibodies (20 µg ml⁻¹) were incubated for 1.5 h before the addition of neurons. Each microscope field covered an area of 3 mm². Mean ± s.e.m. from 3–12 experiments are shown. Chromatofocusing is described in the Fig. 2 legend and illustrated in Fig. 2b. For chromatography on hydroxylapatite, immunoreactive material was eluted from a Bio-Gel HPHT (Bio-Rad) column with a gradient of sodium phosphate (10–350 mM, in 4M urea, 0.1% β-mercaptoethanol and 10 mM CaCl₂, pH 6.9). For immunofluorescence, mAb D5 was coupled to Affigel HZ (Bio-Rad); s-laminin was eluted with 0.1 M glycine-HCl, pH 2.8. Non-s-laminin polypeptides prominent in the hydroxylapatite and D5-agarose eluates were different from those detectable by silver staining in the chromatofocusing peak. Preparation of s-laminin fusion proteins in the pET vector⁴⁰ will be described elsewhere (D.D.H., B. Porter, J.P.M. and J.R.S., in preparation). Neurons did not adhere at levels above background to a non-s-laminin fusion protein or to a pET vector control. C14 is a mouse monoclonal antibody that recognizes a non-s-laminin BL antigen⁶.

(RYVVLPRPVCFEKGMNYTVR) inhibits adhesion of some cells and binding of heparin to laminin²⁶; s-laminin is identical in 11 of 20 positions (RVLVFPRLPVCLEPGLSYKLLK). A hexapeptide from domain V (LGTIPG) inhibits binding of a cellular receptor to laminin (R. Mecham, personal communication); the sequence of s-laminin is RGTVPGL in the corresponding position. Finally, a neurite outgrowth-promoting site on laminin has been localized to a region near the C terminus of B1 and/or B2^{27,28}; this domain is one of the least conserved between s-laminin and the laminin subunits (Fig. 4b). We do not yet know whether these differences conserve or abolish adhesive function.

Database searches (Genbank and NBRF) did not reveal any striking similarities of s-laminin with proteins other than laminin, although short regions of homology between several proteins and the repeats in domains I–III and V were detected, as noted previously for laminin^{19–22}. More detailed comparisons failed to reveal extensive homologies between s-laminin and other neuroactive proteins that are associated with BLs under some circumstances—fibronectin²⁹, entactin³⁰, collagen IV³¹, NCAM³², L1³³ and tenascin³⁴. S-laminin is also distinct from other proteins concentrated at the neuromuscular junction—acetylcholine receptor subunits³⁵, the 43K RAPsyn protein³⁶, acetylcholinesterase³⁷ (or its collagenous subunit³⁸), or agrin³⁹ (the agrin N-terminal sequence was communicated by M. Smith and U. J. McMahan). The possibility that s-laminin is the rat equivalent of laminin B1 can be dismissed because of the limited amino-acid sequence identity (Fig. 4b) and because a laminin B1 cDNA (obtained from B. Hogan; see ref. 18) and a s-laminin cDNA encoding equivalent domains (I and II) recognize distinct restriction fragments on Southern blots of genomic DNA (data

not shown). Thus, s-laminin is apparently a hitherto undescribed component of BL.

Neuronal adhesion to recombinant s-laminin

The cDNA clone RK65-6 encodes the C-terminal 40% of s-laminin. This sequence was subcloned into the pET expression vector⁴⁰ to produce a protein consisting of an 80K s-laminin fragment fused to a 10 amino-acid phage peptide. When tested in the cell-binding assay described above, this fusion protein mediated adhesion of ciliary neurons to substrata; adhesion was blocked by mAbs C4 and D5 (Table 1, series B). Thus, s-laminin contains at least one site that neurons recognize.

Conclusions

Starting with an antiserum that stained synaptic sites in muscle⁵, we have identified a laminin-like component of synaptic BL with which motoneurons can interact. We undertook this work hoping to find factors that regenerating motor axons recognize at original synaptic sites, and our results support the candidacy of s-laminin for this role. The availability of s-laminin cDNAs will now allow critical tests of this hypothesis: effects of recombinant s-laminin on neuronal behaviour can be assessed and antisera to recombinant s-laminin can be tested for their ability to perturb axonal recognition of synaptic BL *in vitro*¹⁵ and *in vivo*. In addition, recombinant s-laminin, and antisera to it, will be useful reagents for probing the function of s-laminin in non-muscle BLs.

The homology of s-laminin to laminin is interesting in two respects. First, it provides evidence for the existence of a laminin gene family that extends beyond the genes for the three laminin subunits. Some other extracellular matrix molecules (for example, the collagens), and some of their cellular receptors (for example, the integrins) are encoded by families of related genes^{41,42}, and the specificity of cell-matrix interactions may result in part from the differential expression of individual members of such families. In this context, it will be important to determine whether s-laminin-like homologues of laminin A and/or B2 subunits exist, whether other laminin-like genes exist, whether s-laminin can substitute for laminin subunits to form chimaeric complexes and whether any effects previously attributed to laminin are actually mediated by s-laminin. Second, it has recently become clear that laminin is a major axon outgrowth-promoting component of extracellular matrices in the peripheral nervous system and that neurons have several laminin receptors⁹. The results presented here raise the possibility that laminin itself is but one of a group of related molecules that could act together to regulate axonal behaviour in complex ways. In muscle in particular, laminin-rich pathways might provide a favourable terrain for regenerating axons to follow, whereas s-laminin at original synaptic sites might direct axons to stop growing and/or to differentiate into nerve terminals. □

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1. Tello, F. *Trab. Lab. Invest. biol. Univ. Madr.* **5**, 117–149 (1907).
2. Bennett, M. R. & Pettigrew, A. G. *Cold Spring Harb. Symp. quant. Biol.* **40**, 409–424 (1975).
3. Sanes, J. R., Marshall, L. M. & McMahan, U. J. *J. Cell Biol.* **78**, 176–198 (1978).
4. Glicksman, M. & Sanes, J. R. *J. Neurocytol.* **12**, 661–671 (1983).
5. Sanes, J. R. & Hall, Z. W. *J. Cell Biol.* **83**, 357–370 (1979).
6. Sanes, J. R. & Chiu, A. Y. *Cold Spring Harb. Symp. quant. Biol.* **48**, 667–678 (1983).
7. Anderson, M. J. & Fambrough, D. M. *J. Cell Biol.* **97**, 1396–1411 (1983).
8. Fallon, J. R., Nitkin, R. M., Reist, N. E., Wallace, B. G. & McMahan, U. J. *Nature* **315**, 571–574 (1985).
9. Sanes, J. R. *Rev. Neurosci.* **12**, 521–546 (1989).
10. Hunter, D. D., Sanes, J. R. & Chiu, A. Y. *Soc. Neurosci. Abstr.* **13**, 375 (1987).
11. Eldridge, C. F., Sanes, J. R., Chiu, A. Y., Bunge, R. P. & Cornbrooks, C. J. *J. Neurocytol.* **15**, 37–51 (1986).
12. Fawcett, D. W. *Histology* (W. B. Saunders, Philadelphia, 1986).
13. Timpi, R. & Dziadek, M. *Int. Rev. exp. Path.* **29**, 1–112 (1986).
14. Pilar, G., Landmesser, L. & Burstein, L. *J. Neurophysiol.* **43**, 233–254 (1980).
15. Covault, J., Cunningham, J. M. & Sanes, J. R. *J. Cell Biol.* **105**, 2479–2488 (1987).
16. Martin, G. R. & Timpi, R. A. *Rev. Cell Biol.* **3**, 57–85 (1987).
17. Barlow, D. P., Green, N. M., Kurkinen, M. & Hogan, B. L. M. *EMBO J.* **3**, 2355–2362 (1984).
18. Sasaki, M., Kato, S., Kohno, K., Martin, G. R. & Yamada, Y. *Proc. natn. Acad. Sci. U.S.A.* **84**, 935–939 (1987).
19. Pikkariainen, T. et al. *J. biol. Chem.* **262**, 10454–10462 (1987).
20. Sasaki, M. & Yamada, Y. *J. biol. Chem.* **262**, 17111–17117 (1987).
21. Pikkariainen, T., Kallunki, T. & Tryggvason, K. *J. biol. Chem.* **263**, 6751–6758 (1988).

22. Durkin, M. E., Bartos, B. B., Liu, S.-H., Phillips, S. L. & Chung, A. E. *Biochemistry* **27**, 5198–5204 (1988).
23. Sasaki, M., Kleinman, H. K., Huber, H., Deutzmann, R. & Yamada, Y. *J. biol. Chem.* **263**, 16536–16544 (1988).
24. Montell, D. J. & Goodman, C. S. *Cell* **53**, 463–473 (1988).
25. Graf, J. *et al. Biochemistry* **26**, 6900–6904 (1987).
26. Charonis, A. S. *et al. J. Cell Biol.* **107**, 1253–1260 (1988).
27. Edgar, D., Timpl, R. & Thoenen, H. *EMBO J.* **3**, 1463–1468 (1984).
28. Engvall, E. *et al. J. Cell Biol.* **103**, 2457–2465 (1986).
29. Kornblitt, A. R., Umezawa, K., Vibe-Pederson, K. & Baralle, F. E. *EMBO J.* **4**, 1755–1759 (1985).
30. Durkin, M. E. *et al. Proc. natn. Acad. Sci. U.S.A.* **84**, 1570–1574 (1987).
31. Wood, L., Theriault, N. & Vogel, G. *FEBS Lett.* **227**, 5–8 (1988).
32. Santoni, M. J. *et al. Nucleic Acids Res.* **15**, 8621–8641 (1987).
33. Moos, L. *et al. Nature* **334**, 701–703 (1988).
34. Pearson, C. A., Pearson, D., Shibahara, S., Hofsteenge, J. & Chiquet-Ehrismann, R. *EMBO J.* **7**, 2977–2981 (1988).
35. Kubo, T. *et al. Eur. J. Biochem.* **149**, 5–13 (1985).
36. Frail, D. E. *et al. J. biol. Chem.* **263**, 15602–15607 (1988).
37. Schumacher, M. *et al. Nature* **319**, 407–409 (1986).
38. Lee, S. I., Heinemann, S. & Taylor, P. *J. biol. Chem.* **257**, 12283–12291 (1982).
39. Nitkin, R. M. *et al. J. Cell Biol.* **105**, 2471–2478 (1987).

40. Rosenberg, A. H. *et al. Gene* **56**, 125–135 (1987).
41. Mayne, R. & Burgeson, R. E. (eds) *Structure and Function of Collagen Types* (Academic, New York, 1987).
42. Buck, C. A. & Horwitz, A. F. *Rev. Cell Biol.* **3**, 179–205 (1987).
43. Lagenaur, C. & Lemmon, V. *Proc. natn. Acad. Sci. U.S.A.* **84**, 7753–7757 (1987).
44. Covault, J. & Sanes, J. R. *J. Cell Biol.* **102**, 716–730 (1986).
45. Hunter, D. D. & Nathanson, N. M. *J. Neurosci.* **6**, 3739–3748 (1986).
46. Towbin, H., Staehlin, T. & Gordon, J. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4350–4354 (1979).
47. VonHeijne, G. *Eur. J. Biochem.* **133**, 17–21 (1983).
48. Huynh, T. V., Young, R. A. & Davis, R. N. in *DNA Cloning: A Practical Approach* (ed. Glover, D. M.) (IRL, Oxford, 1985).
49. Buonanno, A., Mudd, J., Shah, V. & Merlie, J. P. *J. biol. Chem.* **261**, 16451–16458 (1986).
50. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).

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LETTERS TO NATURE

Submillisecond optical pulsar in supernova 1987A

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WE have detected an optical pulsar with frequency $f = 1,968.629$ Hz at the location of supernova 1987A in the Large Magellanic Cloud. The brightness of the pulsed light increased from magnitude 19 to 18 during a 7-h observation period starting on 18.1 January 1989 UT, in observations taken using the 4-m telescope at Cerro Tololo Inter-American Observatory. The frequency of the pulsar during this same period varied in a nearly sinusoidal manner, with an amplitude of 1.5×10^{-3} Hz and a period of 8 h. If this is interpreted as the result of a binary orbit, it suggests that there may be a Jupiter-sized object orbiting the pulsar at a distance of 10^6 km. No significant pulsations were seen in data taken with the same detection system at the 100" telescope at Las Campanas Observatory on 31 January UT, indicating that the pulsar had dropped below our detection limit of magnitude 20. Significant second ($2f$) and third ($3f$) harmonics were also observed.

The detection of the optical pulsar¹ in the supernova 1987A represents the culmination of a search program begun in March 1987, consisting of 37 separate observations from three different observatories². A detector was used that was designed to allow for a pulsar search in the background of the bright light of the supernova. In the detector system, the current from a silicon photodiode was integrated over an interval of 2×10^{-4} s, digitized and written to tape. Manufacturer specifications for the photodiode, Hamamatsu model S1188-06, report the quantum

efficiency of the silicon photodiode peaking at 70% at $0.6 \mu\text{m}$, and greater than 50% between $0.4 \mu\text{m}$ and $0.9 \mu\text{m}$; it averages about 60% over this range. No filter was used for the observations. The aperture size was 13 arcsec.

The analysis of the data follows that of Middleditch and Cordova³. We use a modified Fourier analysis⁴, which differs from the standard Fourier transform by allowing the period of the expected signal to change with time, because the frequency of a pulsar may change significantly during an observation. We then searched the resulting power spectrum for nonstatistical peaks in the frequency range from 0.003 Hz to the Nyquist limit of 2,500 Hz (half the sampling frequency).

The data for 18 January were split into 15 independent contiguous sets. Except for three incomplete data sets, each contains 8 million (2^{23}) 200- μs samples and covers just under $\frac{1}{2}$ hour (1,677 s). Each set was analysed independently to find the frequency and frequency derivative that maximize the peak in the power spectrum. The results are listed in Table 1, which also includes the power found in the aliased second ($2f$) and third ($3f$) harmonics. The times given are the midpoints of each data set. The peak frequencies are shown as a function of time in Fig. 1a; shown in Fig. 1b are the same data after correction

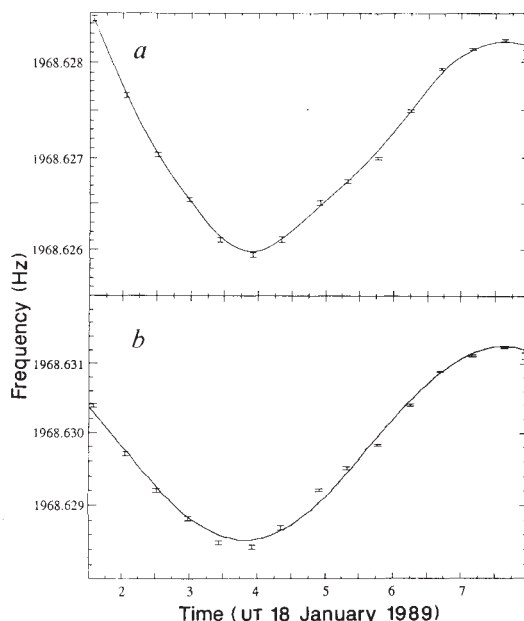


FIG. 1 a, Observed frequency versus time, for the 7-h run on 18 January 1989. The solid line is a guide to the eye. b, Same data, corrected for the Doppler shift resulting from the rotation of the Earth and its motion around the Sun (barycentric coordinates). The solid line is a sine function with best-fit values for amplitude, frequency, phase and offset.

TABLE 1 Observational data for the SN1987A pulsar

UT (h)	Frequency*	δf †	df/dt ‡	Power§	σ	2f power¶	σ	3f power	σ
1.57	1,968.630387	25	-5.4	1,148	13	3,948	7		
2.04	1,968.629710	24	-4.2	1,334	14	3,417	6		
2.51	1,968.629197	27	-3.1	941	12	3,977	7		
2.97	1,968.628823	25	-2.2	1,176	13				
3.43	1,968.628496	25	0.36	1,058	13	1,676	4		
3.92	1,968.628438	28	0.75	903	11				
4.34*	1,968.628700	32	1.4	830	13	3,877	7		
4.90*	1,968.629206	25	0.5	1,548	19	4,094	7		
5.32	1,968.629513	17	2.0	2,188	18	8,689	9		
5.77	1,968.629833	16	3.1	3,049	21	6,042	8	19,235	6.1
6.25	1,968.630407	14	4.6	4,250	25	7,564	9		
6.70	1,968.630888	11	1.6	7,191	31	24,158	15	14,457	5.5
7.16	1,968.631135	10	1.8	8,399	34	31,914	18	10,835	4.9
7.64	1,968.631253	9	-1.3	9,745	37	43,528	21	31,069	8.2
7.95*	1,968.631121	48		3,675	20	7,948	9	19,677	6.1

* In Hz, barycentric coordinates. † Statistical uncertainty in the frequency determination, in units of 1×10^{-6} Hz. ‡ In 10^{-7} Hz s^{-1} . § Spectral power. The signal power is proportional to the square root of this. || Number of standard deviations, indicating the statistical significance of the spectral power in the previous column. ¶ Spectral power for the second and third harmonics was calculated by compensating for the losses from aliasing and from the response of the front-end amplifier for the photodetector. * Values of power are low because data did not fill entire interval.

for the Doppler shift resulting from the rotation of the Earth and its motion around the Sun. The error bars shown are $\pm 1\sigma$, determined by the run duration and the power in the peak. After the Doppler correction, the modulation exhibits a sinusoidal behaviour. A least-squares fit to a sine curve gave a solution with an amplitude of 1.36×10^{-3} Hz, a period of 7.6 h and a central frequency of $f_0 = 1,968.6299$ Hz; this fit is shown as the solid line in Fig. 1b. Also allowing a constant frequency derivative $(df/dt)_0$ in the fit gives an amplitude of 1.77×10^{-3} Hz, a period of 8.8 h, and $(df/dt)_0 = -5.3 \times 10^{-8}$ Hz s^{-1} . We conclude that the period of the modulation is 8 ± 1 h, the amplitude is $(1.5 \pm 0.3) \times 10^{-3}$ Hz, $-(df/dt)_0 < 10^{-7}$ Hz s^{-1} and $(dP/dt)_0 < 3 \times 10^{-14}$ s s^{-1} . The residuals after the two fits were $(6-8) \times 10^{-5}$ Hz (r.m.s.); these are statistically significant when compared with uncertainties in frequency (typically $(1-3) \times 10^{-5}$ Hz).

Twelve minutes after the end of the 7-h observation of SN1987A, the telescope was pointed at the globular cluster NGC3201 (RA $10^h 17^m$, $\delta -46^\circ$) for a half-hour of observation. No significant periodic signal was found in the globular-cluster data. We note that the pulsar signal was strongest just before these globular-cluster observations.

Each of the 8-million-point transforms showed a significant signal at the aliased frequency for the second harmonic, and five transforms (taken near the end of the observation, when the pulsar was brightest) showed a significant signal at the aliased frequency of the third harmonic. The frequency and intensity of the harmonics were modulated in the same manner

as the fundamental, if one takes into account the frequency inversion expected for the aliased signals. The phase coherence of the pulsations was such that they could be tracked to an integral number of cycles across the individual data sets.

We calculate the magnitude of the pulsar by comparison with a calibration run on the Crab pulsar (PSR0531+21) performed one year earlier, on 18 January 1988. We find a broad-band magnitude varying slowly over 7 h from mag 19 to 18 (Fig. 2), with a very significant rise in brightness from the beginning to the end of the run. The maximum magnitude observed was seven magnitudes dimmer than that of the supernova (which was magnitude 11 on 18 January); this is roughly the same as the magnitude difference between the Crab pulsar (magnitude 16.6) and the Crab nebula (magnitude 8.6). Despite a threefold increase in brightness, the ratio of the power emitted in the three harmonics is constant to within 40%. The ratio of amplitudes of the first three harmonics, for the run at 7.64 UT (the run with the best statistical significance) was 1:1.8:1.6. For comparison, in a short run on the Crab pulsar we observed the amplitudes of the first three harmonics to be in the ratio 1:1.8:1.1. Thus the signal shape is basically similar to the double-peaked light curve of the Crab.

It appears unlikely that we are observing two pulses per rotation of a pulsar whose rotation frequency is 984 Hz. No subharmonic was present at a level greater than 3% of the amplitude of the fundamental. If there were two pulses per rotation, they would have to be separated by exactly half of the rotation period and their shapes would have to be the same.

In the 36 runs made previous to the observation of the pulsar², the typical 90% confidence upper limit on the fraction of optical light that is pulsed is 2×10^{-4} of the total light from the supernova. The 90% figure is based on the lack of any peaks in the power spectrum more than 27.3 times the average local spectral power level S_{loc} at nearby frequencies; typically, the highest peaks actually observed were 20 times S_{loc} . (The probability that a random fluctuation will yield a peak with spectral power S_1 is $\exp(-S_1/S_{loc})$.) The range of these limits is set by the observation conditions and the noise at particular frequencies. Before January 1989, our faintest limit was $m = 20.6$, made on 27 August 1988, also at CTIO. On the evening of the detection, the observed spectral power for the fundamental in the 8-million-point transforms was $S_1 = 66 \times S_{loc}$ at its weakest, and as much as $S_1 = 699 \times S_{loc}$ at its strongest. The statistical significance of the peaks corresponding to these spectral power levels is shown in Table 1, and varies from 11 to 37 standard deviations. Thus each of the 15 runs taken on 18 January showed a signal of much greater significance than had been seen on any of the previous 36 runs over the preceding two years.

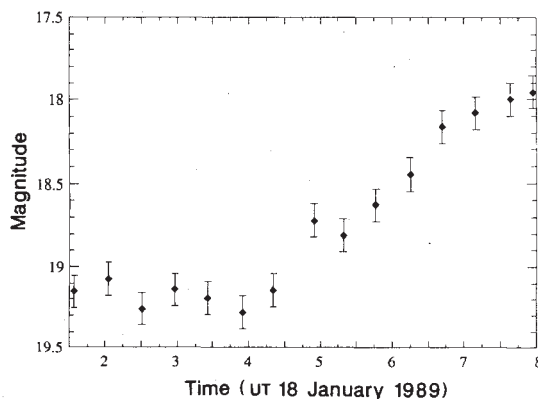


FIG. 2 Magnitude versus time for the 1,968-Hz fundamental frequency, for the same run as in Fig. 1. In addition to the uncertainties shown, there may be a systematic uncertainty of ± 0.5 magnitude for each point.

Given that the magnitude changed from 19 to 18 over the 7-h period on 18 January, it is not too surprising (although disappointing) that the brightness dropped below our detection threshold of 20th magnitude on 31 January. It is possible that the observed brightness variation was due to obscuring material in the expanding supernova shell.

In view of the lack of confirmation so far of the pulsations reported here, it seems useful to summarize the evidence for their proposed origin. They are extremely strong, and are clearly real; the question is whether they originate in the supernova or are merely instrumental. They are unlike anything seen in 36 previous runs using the same instrumentation over the preceding two years; a number of artefacts in the data have been seen over this period, but these pulsations are unprecedented in their strength and their behaviour. Taking into account the 8-h sinusoidal variation, they are phase-coherent over the entire 7 h of observation, so that an integral number of cycles for the fundamental frequency can be counted unambiguously between sets of data. The pulsations are as narrow in $(f, df/dt)$ space as would be a δ -function for such a discrete Fourier transform, which implies an extremely good time-keeping mechanism. In addition to the fundamental frequency, evidence is seen for two higher harmonics which track the fundamental in frequency and intensity. The pulsations are not seen in data taken on another object immediately following the supernova observation. The observed frequency modulation is a good fit to a sine wave only when transformed to barycentric coordinates. The ratio of harmonics is similar to that seen in the Crab pulsar.

With a 0.51-ms period, the pulsar is spinning three times faster than any other known pulsar. For a nominal neutron-star radius of 10 km, the velocity of the surface is 40% of the speed of light. The radius of the light cylinder is only 24 km, as opposed to 1,600 km for that of the Crab pulsar. Assuming a nominal mass of $1 M_{\odot}$, a uniform density and a spherical shape, the rotational energy is 6×10^{52} erg, which is comparable to the entire energy output from the explosion of SN1987A (the shape would, however, be highly non-spherical, because the centrifugal force is comparable to the gravitational force). This rotational energy, together with our limit on the period derivative ($dP/dt < 3 \times 10^{-14} \text{ s s}^{-1}$) means that $dE/dt < 7 \times 10^{42} \text{ erg s}^{-1}$.

The small value of dP/dt suggests that this pulsar is still very close to its initial frequency. Friedman and coworkers^{5,6} have shown that the very existence of a submillisecond pulsar rules out most of the equations of state that have been proposed for neutron stars. Only a very soft equation of state allows the high central compression of matter near the core of the star that is required to provide the gravitational binding necessary to overcome the centrifugal force.

Even a small deviation from axial symmetry in the mass distribution would yield considerable gravitational radiation. If we assume an asymmetric element of mass δm on the surface, yielding a quadrupole moment Q that changes with the rotation of the pulsar, then the energy released as gravitational radiation is approximately

$$-\frac{dE}{dt} \approx \frac{G\omega^6 \delta m^2 R^4}{45c^5} = 10^{54} (\delta m / M_{\odot})^2 \text{ erg s}^{-1}$$

where ω and R are the angular frequency and radius of the pulsar, respectively. Our limit of $-dE/dt < 7 \times 10^{42} \text{ erg s}^{-1}$ gives a limit of $\delta m < 3 \times 10^{-6} M_{\odot}$. Compared with the Crab pulsar, the gravitational radiation expected on the Earth from comparable mass irregularities is greater by a factor of $(\omega/\omega_{\text{Crab}})^6 / (D/D_{\text{Crab}})^2$, that is, 10^8 times larger. Thus the pulsar may be a candidate for the detection of gravitational radiation, although for maximum sensitivity the detection must take into account the modulated frequency behaviour, perhaps by using an ephemeris derived from the optical signal.

If we assume that the energy loss rate from the pulsar is less than that radiated by the supernova ($\sim 10^{38} \text{ erg s}^{-1}$), then we

conclude that the pulsar must have a relatively weak magnetic field B . This can be seen easily by comparison with the Crab pulsar, which coincidentally also loses power at the rate of $10^{38} \text{ erg s}^{-1}$. If we use the scaling law for dipole emission with radiated power proportional to $B^2 \omega^4$, the magnetic field would be lower than that of the Crab pulsar by the square of the frequency ratio, $(1,968/30.2)^2 = 4 \times 10^3$. Taking $B_{\text{Crab}} = 4 \times 10^{12}$ gauss, we find the field for the SN1987A pulsar to be $B = 10^9$ gauss.

The observed 8-h frequency modulation of the pulsar could be due to an intrinsic change in the rotation frequency of the pulsar, or to a Doppler shift. If the change is intrinsic, it could be due to a periodic change by 10^{-6} in the moment of inertia of the pulsar, or to oscillations between two components of the pulsar (such as a hard crust and a superfluid core). In the two-component model, the rotation angle between the two components oscillates with a period of 8 h and an amplitude of $(1.4 \times 10^{-3} \text{ Hz}) \times (8 \text{ h}) = \pm 40$ radians $= \pm 6.4$ turns, that is, about 13 turns peak-to-peak. Because of the great amount of energy stored in the pulsar spin, the forces required to drive such oscillations are enormous. A successful model must account for the relatively long period of 8 h while maintaining sufficient linearity under 13 turns to reproduce the observed sine-like variation.

The modulation could also be explained as a Doppler shift resulting from motion of the pulsar in a binary system. The observed frequency modulation is similar to that seen in PSR1957+20, a 1.5-ms pulsar with a binary companion in a 9.16-h orbit⁷. The frequency modulation of $1.4 \times 10^{-3} \text{ Hz}$ for the SN1987A pulsar implies a velocity (in km s^{-1}) of $0.2/\sin i$, where i is the inclination angle. The nearly sinusoidal behaviour implies a nearly circular orbit; the orbital frequency is $\Omega_0 = 2.2 \times 10^{-4} \text{ s}^{-1}$; the orbital radius of the pulsar is $r_0 = v/\Omega_0 = (10^3 \text{ km})/\sin i$. From Kepler's law, the distance between the pulsar and its companion is $(GM/\Omega_0^2)^{1/3} = 1.3 \times 10^6 \text{ km}$. The mass of the companion is then $10^3/(1.3 \times 10^6 \sin i) = 8 \times 10^{-4} M/(\sin i)$, which is comparable to the mass of Jupiter for $M = M_{\odot}$. The mass function is thus about $2.5 \times 10^{-10} M_{\odot}$.

This orbit would have been well within the radius of the blue giant progenitor star; it could not have survived the supernova explosion in such an orbit, so it would have to have been placed into orbit with the pulsar after the explosion. Perhaps it was left over from the core collapse, or ejected from the spinning pulsar, and then circularized by nearby orbiting material. We note that the pulsar has almost the maximum possible rotational velocity, and that the angular momentum of the companion is similar to that of the pulsar. The deviations of the oscillations from sinusoidal are statistically significant, and may indicate departures from a circular orbit, perhaps as a result of additional debris orbiting the pulsar, or they may indicate intrinsic changes in the pulsar rotation rate. The evolution of the sinusoidal modulation will be of great interest as a possible probe of the region 10^6 km from the pulsar.

Note added in proof. The pulsar was not seen in later observations on 13 February (Siding Springs 2.3-m telescope), and 16, 20 and 21 February (Las Campanas 100" telescope), all with limits fainter than 20th magnitude. □

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1. Middleditch, J. *et al.* *IAU Circ.* No. 4735, (1989).
2. Pennypacker, C. *et al.* *Astrophys. J.* (in the press).
3. Middleditch, J. & Cordova, F. A. *Astrophys. J.* **255**, 585–595 (1982).
4. Middleditch, J. & Kristian, J. *Astrophys. J.* **279**, 157–162 (1984).
5. Friedman, J. L., Ipser, J. R. & Parker, L. *Nature* **312**, 255–257 (1984).
6. Friedman, J. L., Imamura, J. N., Durisen, R. H. & Parker, L. *Nature* **336**, 560–562 (1988).
7. Fruchter, A. S., Stinebring, D. R. & Taylor, J. H. *Nature* **333**, 237–239 (1988).

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A numerical experiment on the chaotic behaviour of the Solar System

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LAPLACE and Lagrange made an essential contribution to the study of the stability of the Solar System by proving analytically that, to first order in the masses, inclinations and eccentricities of their orbits, the planets move quasiperiodically. Since then, many analytic quasiperiodic solutions have been sought to higher order¹⁻¹⁰. I have recently constructed an extensive analytic system of averaged differential equations containing the secular evolution of the orbits of the eight main planets, accurate to second order in the planetary masses and to fifth order in eccentricity and inclination, and including corrections from general relativity and the Moon⁸⁻¹⁰. Here I describe the results of a numerical integration of this system, extending backwards over 200 million years. The solution is chaotic, with a maximum Lyapunov exponent that reaches the surprisingly large value of $\sim 1/5 \text{ Myr}^{-1}$. The motion of the Solar System is thus shown to be chaotic, not quasiperiodic. In particular, predictability of the orbits of the inner planets, including the Earth, is lost within a few tens of millions of years. This does not mean that after such a short timespan we will see catastrophic events such as a crossing of the orbits of Venus and Earth; but the traditional tools of quantitative celestial mechanics (numerical integrations or analytical theories), which aim at unique solutions from given initial conditions, will fail to predict such events. The problem of the stability of the Solar System will have to be set up again, and the qualitative methods initiated by Poincaré definitely need to replace quantitative methods in this analysis.

After the fundamental work of Laplace and Lagrange, it was assumed that the motions of the planets were quasiperiodic, and efforts in celestial mechanics in the nineteenth century were devoted to the construction of quasiperiodic solutions for all the bodies of the Solar System using perturbation techniques. This approach was questioned when Poincaré proved that, if it is possible to expand formally the solutions of the planet motions in infinite series, then in general these series are not convergent^{11,12}.

The KAM theory¹³ nevertheless proved that if the perturbation is not too large (that is, if the masses, eccentricities and inclinations of the planets are sufficiently small), then a large set of initial conditions leads to quasiperiodic solutions¹³. It was not proved that the Solar System fulfilled these requirements. In fact, the Solar System is much too complicated, with far too many parameters, to obtain mathematical results on its stability at present (the mathematical knowledge of dynamical systems does not extend very far beyond two degrees of freedom).

To decide whether the initial conditions of the Solar System lead to a quasiperiodic or a chaotic solution, we have two approaches available: perturbation techniques and numerical integration. Improvements in computer speeds have enabled several numerical integrations on the long-term evolution of the Solar System to be made¹⁴⁻¹⁹. All of these deal with the outer Solar System (Jupiter to Pluto) and span from 500,000 years (ref. 14) to almost 10^9 years (in the latest integration¹⁹). One major result is numerical evidence for the chaotic motion of Pluto¹⁹ from computation of its maximum Lyapunov exponent, which was found to be about $1/20 \text{ Myr}^{-1}$. This result gives the limit of predictability for the motion of Pluto, but as Pluto does not significantly perturb the rest of the Solar System, it yields no information on the limit of predictability of the essential part of the Solar System.

The direct numerical integration of the Solar System is

reliable, but cannot as yet take into account the inner planets (Mercury, Venus, the Earth and Mars), for which orbital motion is too rapid. Here I use perturbation theory to remove the short periods from the motion equations; the averaged equations of the Solar System are then numerically integrated by Adam's method using a very large (500 yr) step size. I have constructed an extensive analytical differential system that gives the secular evolution of the orbits of the eight main planets of the Solar System, accurate up to second order with respect to the planetary masses and to fifth order in eccentricity and inclination. Corrections for the secular effects of the Moon and relativity are included, and a final differential system of about 150,000 polynomial terms⁹ is obtained. The accuracy of the solution obtained by numerical integration of this system was checked by comparison with the complete numerical ephemeris of the Solar System DE102 (ref. 20) over the range of DE102, or 44 centuries¹⁰. This system was then numerically integrated over 30 Myr and its solution treated with a modified Fourier analysis to derive a solution of a quasiperiodic form over 10 Myr (ref. 21). At the same time, I found that there were many resonances in the long periods of the inner Solar System which prevented a good convergence of the solutions, suggesting that the inner Solar System is unstable²² (similar problems of convergence have been encountered to a lesser degree by Nobili *et al.*²³ in their 100-Myr integration of the outer planets). The secular system has now been improved slightly by a small correction obtained by adjustment of three of the diagonal terms of the linear part of the secular system in order to take partially into account the third-order contribution with respect to the masses. This small change essentially improved the solution for the outer planets.

I have integrated the full system over 200 Myr. Applying my Fourier techniques to the numerical output, it was not possible to obtain a quasiperiodic solution valid over the whole timespan: when applied to different intervals of the timespan, the Fourier analysis showed significant variations of the main secular

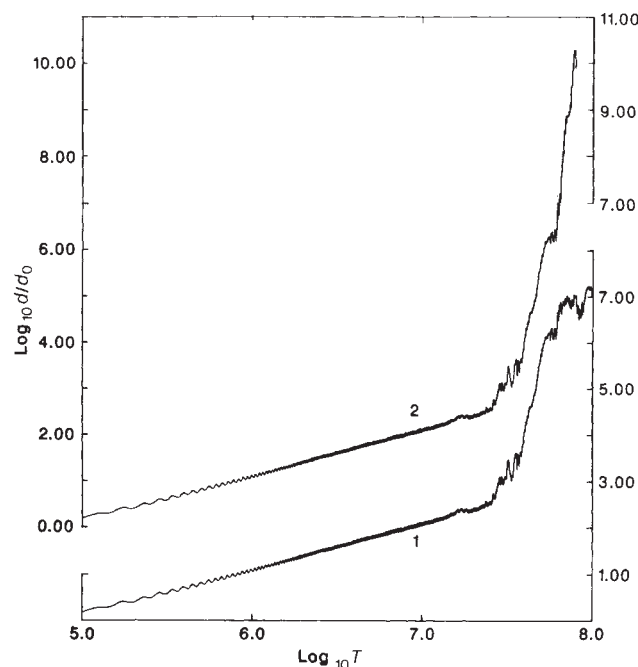


FIG. 1 Divergence of two nearby orbits. We plot $\log_{10}(d/d_0)$ versus $\log_{10} T$ (where T is time in years) over 100 Myr. At the beginning, the distance of two nearby orbits increases at a nearly linear rate. After about 30 Myr, an exponential divergence of the orbits dominates. The initial relative distance in the phase space is $d_0 = 10^{-7}$. In the absence of renormalization, the distance is bounded after ~ 60 Myr (curve 1). With a renormalization every 40 Myr, the second orbit stays close to the first and the cumulated distance is not bounded (curve 2).

frequencies of the inner planets, whereas for the outer planets, secular frequencies were steady (J. L., manuscript in preparation). All these facts suggest that the motion of the Solar System is chaotic.

The Lyapunov exponent measures the exponential rate of divergence of two nearby orbits. In quasiperiodic motion it should be zero. A non-zero Lyapunov exponent indicates chaotic motion^{24,25}. The maximum Lyapunov exponent was estimated by computing the distance between two nearby orbits (with relative initial distance $d_0 = 10^{-7}$) in three different ways (Figs 1 and 2): either without renormalization (in this case the distance is bounded after ~ 60 Myr) or with renormalization every 40 Myr or every million years. In the three cases, I found the same Lyapunov exponent with the surprisingly high value of about $1/5 \text{ Myr}^{-1}$.

This result gives a measure of the limit of predictability of my secular system: we have $d(T) \approx d(0) e^{T/5}$, where $d(T)$ is the distance of two close orbits in the phase space at time T in Myr. This shows that it is possible to give a precise solution of the Solar System over 10 Myr but not over 100 Myr (a perturbation as small as 10^{-10} of the initial conditions will lead to 100% discrepancy after 100 Myr). This result is particularly significant for the Milankovitch theory of palaeoclimate and the use of the orbital theory of the Earth as a timescale for changes in climate²⁶.

The chaotic behaviour of the Solar System comes mainly from the secular resonances among the inner planets (Mercury, Venus, the Earth and Mars). Predictions concerning the outer planets may last somewhat longer. I have not computed the maximum Lyapunov exponent of the Solar System with the inner planets excluded but, from the very regular behaviour of the solutions over 200 Myr compared with the inner-planet solutions, it is expected to be much smaller. This would explain why until now, except in the special case of Pluto, there have been no reports of non-zero Lyapunov exponents in the numerical integrations of the outer planets.

This surprisingly high Lyapunov exponent compared with the age of the Solar System needs cautious interpretation. The computation shows that the solutions of our differential system,

with the initial conditions of the Solar System, are chaotic. This differential system is a close approximation to the real Solar System. We can conclude that the motion of the Solar System, and in particular the inner Solar System, is close to being chaotic, but the exact meaning of 'close' is still difficult to evaluate. Using perturbation methods, I have estimated the quality of the approximations used by comparison with the available numerical integrations^{10,21}. This secular theory is more precise than any previously computed but in order to confirm these results, the computations should be repeated with a higher order of approximation. $\square$

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- Hill, G. W. *Astr. J.* **17**(11), 81–87 (1897).
- Brouwer, D. & Van Woerkom, A. J. *J. Astr. Pap. Am. Ephemer.* **13**(2), 81–107 (1950).
- Brumberg, V. A. *Analytical Algorithms of Celestial Mechanics* (Nauka, Moscow, in Russian) (1980).
- Bretagnon, P. *Astr. Astrophys.* **30**, 141–154 (1974).
- Duriez, L. *Astr. Astrophys.* **54**, 93–112 (1977).
- Duriez, L. thesis, Lille (1979).
- Message, P. *J. Celest. Mech.* **26**, 25–39 (1982).
- Laskar, J. thesis, Observatoire de Paris (1984).
- Laskar, J. *Astr. Astrophys.* **144**, 133–146 (1985).
- Laskar, J. *Astr. Astrophys.* **157**, 59–70 (1986).
- Poincaré, H. *Méthodes Nouvelles de la Mécanique Celeste* Vol. 1 (Gauthier-Villars, Paris, 1892).
- Poincaré, H. *Méthodes Nouvelles de la Mécanique Celeste* Vol. 2 (Gauthier-Villars, Paris 1893).
- Arnold, V. *Méthodes Mathématiques de la Mécanique Classique* (Mir, Moscow, 1976).
- Cohen, C. J., Hubbard, E. C. & Oesterwinter, C. *Astr. Pap. Am. Ephemer.* **22**(1), 1–42 (1973).
- Kinoshita, H. & Nakai, H. *Celest. Mech.* **34**, 203–217 (1984).
- Milani, A., Nobili, A. M., Fox, K. & Carpio, M. *Nature* **319**, 386–388 (1986).
- Applegate, J. H., Douglas, M. R., Gursel, Y., Sussman, G. J. & Wisdom, J. *Astr. J.* **92**, 176–194 (1986).
- Carpino, M., Milani, A. & Nobili, A. M. *Astr. Astrophys.* **181**, 182–194 (1987).
- Sussman, G. J. & Wisdom, J. *Science* **241**, 433–437 (1988).
- Newhall, X. X., Standish, E. M. & Williams, J. G. *Astr. Astrophys.* **125**, 150–167 (1983).
- Laskar, J. *Astr. Astrophys.* **198**, 341–362 (1988).
- Laskar, J. in *Proc. 10th ERAM of the IAU* Vol. 3 (ed. Sidichovsky, M.) 95–98 (1987).
- Nobili, A. M., Carpio, M. & Milani, A., *Astr. Astrophys.* (in the press).
- Bennettin, G., Galgani, L., Giorgilli, A. & Strelcyn, J. M. *Meccanica* March 1980, 9–30.
- Froeschle, C. in *Stability of the Solar Systems and its Minor Natural and Artificial Bodies* (ed. Szebehely, V. G.) 265–282 (Reidel, Dordrecht, 1985).
- Berger, A., Imbrie, J., Hays, J., Kukla, G. & Saltzman, B. (eds) *Milankovitch and Climate* (Reidel, Dordrecht, 1984).

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The earliest known solar eclipse record redated

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AN astronomical event recorded on a clay tablet found in 1948 among the ruins of the ancient city of Ugarit, in what is now Syria, was identified 20 years ago as a description of a total solar eclipse that occurred on 3 May 1375 BC^{1,2}. The dating of ancient solar eclipses provides reference points to fix the long-term evolution of angular momentum in the Earth–Moon system³. We have reanalysed the Ugarit eclipse record⁴. A new historical dating of the tablet, and mention in the text of the visibility of the planet Mars during the eclipse as well as the month in which it occurred enables us to show that the recorded eclipse in fact occurred on 5 March 1223 BC. This new date implies that the secular deceleration of the Earth's rotation has changed very little during the past 3,000 years.

Tablet KTU 1.78 was discovered in 1948 among the remains of the Western Palace Archive in Ugarit⁵. The contents of this archive show that it was used over the entire span of the Late Bronze Age III period (around 1350–1175 BC)⁶. However, of all the different genres found in the various archives of Ugarit, only the legal texts and treaties were kept for future reference throughout this period. The other genres were represented only

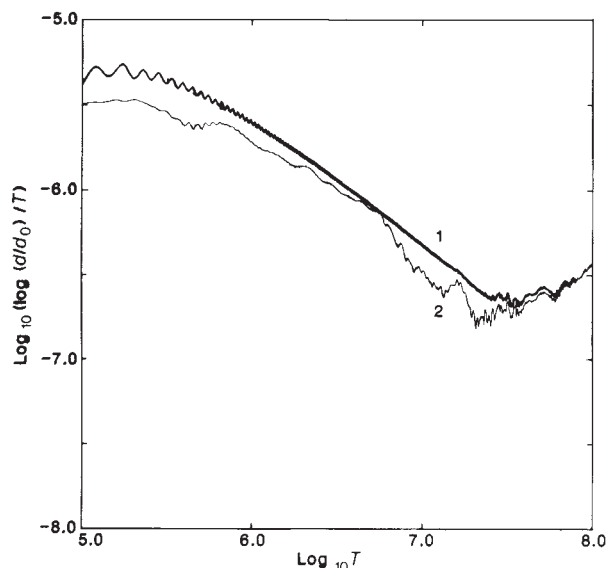


FIG. 2 Computation of the maximum Lyapunov exponent. We plot $\log_{10}(\log(d/d_0)/T)$ versus $\log_{10} T$ (where T is the time in years) over 100 Myr, with renormalization every 40 Myr (curve 1) or with renormalization every million years (curve 2) (the two curves do not coincide for the first million years because of different initial conditions). In the two cases, we obtain the same results for large values of T , which give a maximum Lyapunov exponent of about $10^{-6.7} \text{ yr}^{-1}$ ($1/5 \text{ Myr}^{-1}$) for the secular system in eccentricity and inclination of the Solar System.

by texts dating to the last half-century of Ugarit's existence⁶. Therefore, it is likely that KTU 1.78 dates from around the second half of the thirteenth century or the first quarter of the twelfth century BC.

The text recorded on tablet KTU 1.78 was studied in detail by Sawyer and Stephenson², who were the first to realize that the astronomical event described may refer to a total solar eclipse. The text has recently been studied again by van Soldt⁴. The new translation runs as follows:

(Obverse) "On the... day of the new moon in (the month) *hiyaru* the Sun went down, its gate-keeper was *Ršp*". (Reverse) "Two livers were examined: danger".

The meaning of the phrase "*bit*", left open in the translation above, is problematic. Sawyer and Stephenson rejected the most obvious translation of "*ti*" as "six" because a solar eclipse can occur only close to the beginning of a lunar month. Van Soldt⁴ offers "the sixth hour" as an admittedly speculative possibility. As already pointed out by Sawyer and Stephenson, *Ršp* is probably to be identified with the planet Mars.

The correct translation of the text on the reverse side of the tablet is due to Dietrich *et al.*⁷. Apparently the anxiety caused by the eclipse of the Sun and the sudden appearance of Mars had to be resolved by an explanation through liver divination.

To be able to fix more precisely the date at which the eclipse was observed we need to know the place of the month *hiyaru* in the Ugaritic calendar. The Ugaritic calendar was a lunar one consisting of twelve months, of which ten are known by name. Making use of a collation of a key text in the Damascus Museum by one of us (W.H.v.S., unpublished work), we were able to establish the order of the months⁴. According to de Moor⁸, the year in Ugarit started with the first day of *riš yn*, "the new Moon nearest to the autumn equinox". The reconstructed calendar and its approximate relation to the months in the Julian calendar in 1200 BC are summarized in Table 1. The synchronization to the Julian calendar can only be approximate because by occasional intercalation the beginning of the year was kept close (within about four weeks) to the date of the autumn equinox. We conclude that the first day of *hiyaru* must have fallen in the second half of February or the first half of March.

Based on the considerations above we draw up a list of conditions which an attempted dating of the solar eclipse in KTU 1.78 has to satisfy. (1) The year in which the eclipse took place should preferably be found between 1250 and 1175 BC. (2) The planet Mars must have been visible during the eclipse. (3) The date (the "day of the new Moon" in the month *hiyaru*) has to be set in the second half of February or in the first half of March.

Sawyer and Stephenson² identified four solar eclipses that could have been total in Ugarit during the period 1450–1200 BC: on 14 July 1406 BC, 3 May 1375 BC, 8 January 1340 BC and 5 March 1223 BC (all dates are given according to the Julian calendar). They selected the one that occurred on 3 May 1375 BC as the eclipse referred to in the tablet, based on the fact that it takes place in April–May, the time of year that they associated with the month of *hiyaru*. This association is based on an erroneous identification of *hiyaru* with the Babylonian month *ajjaru*.

Using a recent tabulation⁹ of all solar eclipses observable in the ancient Near East from 3000 BC to AD 0, we find that only

TABLE 1 The order of the months in the Ugaritic Calendar and their relation to the Julian Calendar in 1200 BC

1	<i>riš yn</i>	Sep/Oct	7	<i>hlt</i>	Mar/Apr
2	<i>nql</i>	Oct/Nov	8	<i>gn</i>	Apr/May
3	<i>mgmr</i>	Nov/Dec	9	<i>itb</i>	May/Jun
4	<i>pgrm</i>	Dec/Jan	10	?	Jun/Jul
5	<i>lb'lt</i>	Jan/Feb	11	?	Jul/Aug
6	<i>hyr</i>	Feb/Mar	12	<i>lbtbm</i>	Aug/Sep

those of 1406 BC, 1375 BC and 1223 BC could have come close to being total in Ugarit. Between 1250 BC and 1175 BC the eclipse of 5 March 1223 BC is the only possible candidate.

We must now ask whether Mars was in the sky at the time of this eclipse. By using the series expansions of planetary perturbations published by van Flandern and Pulkkinen¹⁰, we are able to calculate by computer solar, lunar and planetary positions for arbitrary epochs. All time-dependent terms in the solar, lunar and planetary elements listed in the Explanatory Supplement of the Astronomical Ephemeris¹¹ (ESAE) are included in these calculations. The accuracy of the resulting positions is estimated at about one arcmin for dates more recent than 2,000 years ago. For dates after 600 BC the results can be compared with the less accurate tables of Tuckerman¹². The calculated positions usually agree to within 0.1°.

Of the three candidate eclipses mentioned above, the one occurring on 5 March 1223 BC is the only one during which the planet Mars was above the horizon. More significantly, Mars was located at 337.0° ecliptic longitude and −0.8° ecliptic latitude, only 3.5° from the centre of the eclipsed Sun at ecliptic longitude 333.6°.

The observation that Mars was present during the eclipse is a strong indication that the eclipse must indeed have been total. Muller and Stephenson¹³ discuss the visibility of stars and planets during a solar eclipse and conclude that the brightest stars and planets are usually visible during a total eclipse, which is consistent with many eyewitness accounts.

Using formulae given by Allen¹⁴, we have calculated a visual magnitudes for all planets, and all stars brighter than Mars, above the horizon during the eclipse. The results are listed in Table 2. Visual magnitudes for the stars were taken from ref. 15. The data in Table 2 show that Mars was amongst the ten brightest objects in the sky. Its proximity to the eclipsed sun may have decreased its observability because of the coronal glow, but may also have enhanced the likelihood of its observation.

The "day of the new Moon" for the eclipse date does not make sense in a Babylonian-type lunar calendar, for which months began at sunset on the day of first crescent visibility. It fits exactly⁴, however, if we assume an Egyptian-type lunar calendar, for which the month began at sunrise on the day of first crescent invisibility and the second day of the month was named "new crescent day"¹⁶. It is not unexpected to find the Egyptian calendar in Ugarit. We know that it was used in ancient Israel¹⁷, and Egyptian influence in Ugarit during the second millennium BC is attested in several sources¹⁸.

TABLE 2 The brightest stars and planets above the horizon in Ugarit on 5 March 1223 BC at 13°20' Local Mean Time, listed in order of decreasing visual brightness

Star	Name	Az* (degrees)	El* (degrees)	<i>m_v</i> (magnitude)
	Sun/Moon	337	41	
	Venus	22	61	−4.58
α CMa	Sirius	65	2	−1.46
α Lyr	Vega	231	15	0.03
α Aur	Capella	99	63	0.08
β Ori	Rigel	49	20	0.12
α CMi	Procyon	89	13	0.38
α Ori	Betelgeuse	67	29	0.50
	Mercurius	317	20	0.67
α Tau	Aldebaran	51	47	0.85
β Gem	Pollux	105	29	1.14
α Cyg	Deneb	246	35	1.25
	Mars	341	43	1.28

* Positions are given in azimuth and elevation rounded off to full degrees. Azimuth is measured anticlockwise along the horizon from the south (Az = 0°), elevation is measured perpendicular to the horizon (zenith corresponds to El = 90°).

If time was also kept in the Egyptian way (ten day-hours from sunrise to sunset¹⁹) the suggested translation of *btt* as "sixth hour" gains credibility because the eclipse took place at 13^h20^m, whereas sunrise and sunset occurred at 6^h45^m and 17^h45^m Local Mean Time, respectively.

Using the order of the months in Table 1 we find that the beginning of the Ugaritic year, the first day of the month *riš yn*, fell on 7 October 1224 BC, quite close to the actual equinox date of 1 October.

We have investigated the implications of the new eclipse date for the secular deceleration of the Earth's rotation rate. We tested our calculations by comparison with modern data of a number of twentieth-century eclipses²⁰.

To include the effect of the secular accelerations of the Earth and Moon for dates in the past, the ESAE recommends the following expressions for the secular increment in the mean longitude of the Moon, λ_m , and for the difference between Universal and Ephemeris Times:

$$\Delta\lambda_m = -11.22 T^2 \text{ arcsec} \quad (1)$$

$$\Delta T = ET - UT = 24.3 + 72.3 T + 30.0 T^2 \text{ seconds} \quad (2)$$

where T is the time in Julian centuries since 1.0 January 1900. The magnitude of $\Delta\lambda_m$ corresponds to an acceleration rate of the Moon's mean longitude of $\dot{\lambda} = -22.44 \text{ arcsec century}^{-2}$. According to equations (1) and (2), in 1223 BC the effect of the secular accelerations produces large differences in lunar longitude and in time, 3.0° and 8.8 hours respectively.

The standard ESAE value of the lunar acceleration rate is confirmed by recent studies. The McDonald Observatory lunar laser-ranging experiment²¹ gives a value of $\dot{\lambda} = -23.8 \pm 1.5 \text{ arcsec century}^{-2}$, equal, within errors, to the standard value. A re-analysis²² of modern (1717–1973) lunar and planetary observations yields $\dot{\lambda} = -22.2 \pm 0.8 \text{ arcsec century}^{-2}$, which is fully consistent with the lunar laser-ranging result and the standard value.

To accommodate variations in the deceleration rate of the Earth relative to the standard ESAE value we add to ΔT in equation (2) a time parameter δt . If the adopted deceleration rate is too large or too small, δt will systematically decrease or increase going further into the past.

We have analysed the eclipse of 5 March 1223 BC in Ugarit (geographical coordinates 35°37' N, 35°47' E) by calculating its magnitude as a function of time for several values of the parameter δt . Requiring that the eclipse must have been total results in the condition $-3^m \leq \delta t \leq 6^m$. The accuracy of this result is estimated to be about 3 min. Thus the average deceleration of the Earth's rotation rate over the past 3,000 years is well described by the standard ESAE value of the deceleration parameter, and long-term variations (on the scale of centuries) in the deceleration rate over this period have probably been small. This result is of geophysical importance because it helps to establish the nature of the processes, other than the lunar tidal torque²³, affecting the rotation rate of the Earth. □

19. Neugebauer, O. *The Exact Sciences in Antiquity* (Brown Univ. Press, Providence, 1957).
20. Meeus, J., Grosjean, C. C. & van der Leen, W. *Canon of Solar Eclipses* (Pergamon, Oxford, 1966).
21. Dickey, J. O., Williams, J. G. & Yoder, C. P. in *IAU Colloq.* **63**, (ed. Calame, O.) 209–216 (Reidel, Dordrecht, 1982).
22. Krasinsky, G. A., Saramonova, E. Y., Sveshnikov, M. L. & Shveshnikova, E. S. *Astr. Astrophys.* **145**, 90–96 (1985).
23. Wahr, J. *Sky and Telescope* 545–549 (1986).

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Superconductivity at 27 K in fluorine-doped Nd₂CuO₄

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THE recent report¹ of superconductivity in Nd_{1.85}Ce_{0.15}CuO₄ is the first example of a high-transition-temperature (high- T_c) superconductor with electrons as the charge carriers and a formal copper oxidation state of less than +2. All previously characterized copper oxide superconductors are hole-doped, and the copper is formally oxidized. Here we show that substitution of fluorine for oxygen in T'-phase Nd₂CuO₄ provides an alternative route to achieving formal reduction of copper, electron conductivity and superconductivity at temperatures as high as 27 K. This result is unusual, because anionic rather than cationic substitution gives rise to the superconductivity.

We explored several routes to the preparation of Nd₂CuO_{4-x}F_x; the best results were obtained by heating stoichiometric mixtures of CuO, Nd₂O₃ and freshly prepared NdF₃ in air at 900 °C for 14 h followed by pelletizing and heating at 890 °C for 14 h in flowing N₂. A single phase with the T' structure was obtained for samples of nominal composition Nd₂CuO_{4-x}F_x with 0 < x < 0.4. Fluorine analysis was carried out by the Schwarzkopf Microanalytical Laboratory on a sample of nominal composition x = 0.4; the analysed composition was found to be x = 0.28. (Hereafter we will refer always to nominal compositions.) Loss of fluorine could occur in the initial firing step by hydrolysis of NdF₃ to NdOF (ref. 2). After the initial firing in air, X-ray powder-diffraction patterns of the Nd₂CuO_{4-x}F_x samples showed the characteristic lines of the T' structure, with small amounts of NdOF; the NdOF lines disappeared after firing in N₂. Lattice parameter refinements showed that fluorine substitution results in a small contraction (up to 0.2%) of the c axis and a small expansion (up to 0.3%) of the a and b axes; for example, the lattice parameters of the Nd₂CuO_{3.6}F_{0.4} sample were found to be a = 3.961 ± 0.006 Å,

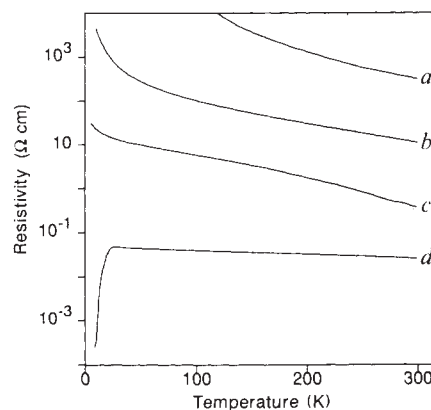


FIG. 1 Electrical resistivity of a, Nd₂CuO₄ prepared in air at 900 °C; b, Same sample after annealing in N₂ at 890 °C for 14 h; c, Sample of nominal composition Nd₂CuO_{3.6}F_{0.4} prepared in air at 900 °C; d, Same fluorinated sample after annealing in N₂ at 890 °C for 14 h.

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1. Stephenson, F. R. *Nature* **228**, 651–652 (1970).
2. Sawyer, J. F. A. & Stephenson, F. R. *Bull. School Oriental African Studies* **33**, 467–489 (1970).
3. Maddox, J. *Nature* **313**, 733 (1985).
4. De Jong, T. & van Soldt, W. H. *Jber "Ex Oriente Lux"* (in the press).
5. Virolleaud, Ch. *Syria* **28**, 25–27 (1951).
6. Van Soldt, W. H. thesis, Univ. of Leiden (1986).
7. Dietrich, M., Lorentz, O. & Sammartin, J. *Ugarit Forsch.* **6**, 464–465 (1974).
8. De Moor, G. *AOAT* **16**, 245 (1971).
9. Kudleck, M. & Mickler, E. H. *Solar and Lunar Eclipses of the Ancient Near East from 3000 BC–0 with Maps*, (Butzon & Bercker Kevelaer, Neukirchen, 1971).
10. Van Flandern, T. C. & Pulkkinen, K. F. *Astrophys. J. Suppl. Ser.* **41**, 391–411 (1979).
11. *Explanatory Supplement to the Astronomical Ephemeris* H. M. Stationery Office, London (1961).
12. Tuckerman, B. *Planetary, Lunar and Solar positions 601 BC–AD 1* (Am. phil. Soc., Philadelphia, 1962).
13. Muller, P. M. & Stephenson, F. R. in *Growth Rhythms and the History of the Earth's Rotation* (eds Rosenberg, G. D. & Runcorn, S. K.) 459–533 (Wiley, London, 1975).
14. Allen, C. W. *Astrophysical Quantities* (Athlone, London, 1973).
15. Hoffleit, D. *Yale Catalogue of Bright Stars*, 3rd revised ed (Yale Univ. Press, New Haven, 1964).
16. Parker, R. A. *The Calendars of Ancient Egypt* (Univ. of Chicago Press, 1950).
17. De Vaux, R. *Les Institutions de l'Ancient Testament* Vol. 1, 271 (Les Editions du Cerf, Paris, 1958).
18. Rainey, A. F. *Bibl. Archeol.* **28**, 109 (1965).

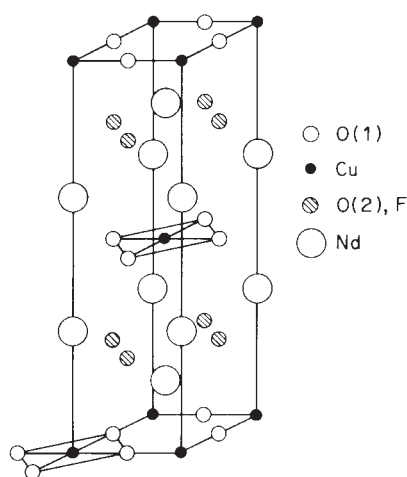


FIG. 2 The T'-Nd₂CuO₄ structure, showing the suggested location of the fluorine atoms in Nd₂CuO_{4-x}F_x.

$c = 12.13 \pm 0.02$ Å. The average Cu oxidation state in samples of Nd₂CuO_{4-x}F_x was determined by iodometric titration³. The unsubstituted material Nd₂CuO₄ was found to have a Cu oxidation state of $+2.00 \pm 0.02$ both before and after firing at 890 °C in nitrogen. The $x=0.4$ sample gave a Cu oxidation state of $+1.89 \pm 0.02$ after the initial firing in air and $+1.76 \pm 0.02$ after firing in N₂ overnight. Combining the fluorine and oxidation-state analyses gives an overall composition of Nd₂CuO_{3.74}F_{0.28}, which is very close to the stoichiometric limit of four anions per Cu. These results indicate that nitrogen annealing at 890 °C results in a greater degree of fluorine substitution in Nd₂CuO_{4-x}F_x compared with air firing, without changing the total anion stoichiometry.

Substitution of F for O was also attempted in the other rare-earth copper oxides M₂CuO₄ (where M represents Pr, Sm, Eu or Gd), all of which have the T' structure. Single-phase materials of nominal composition Pr₂CuO_{4-x}F_x ($0 < x < 0.4$) could be prepared by a method similar to that described above. Pure Sm, Eu and Gd analogues, however, could not be prepared because of increasingly facile reductive decomposition under N₂ to mixtures of the corresponding rare-earth oxides, oxyfluorides and Cu₂O. A similar reductive decomposition under N₂ was observed for Pr₂CuO_{4-x}F_x and Nd₂CuO_{4-x}F_x at temperatures above 950 °C and 900 °C respectively. Recent studies have shown that the cerium-substituted superconductor Nd_{1.85}Ce_{0.15}CuO₄ also decomposes at elevated temperature under N₂.

Figure 1 shows resistivity as a function of temperature for Nd₂CuO_{4-x}F_x (nominal composition $x=0, 0.4$). The sample of Nd₂CuO_{3.6}F_{0.4} fired in air shows a slope that is characteristic of a semiconductor, with conductivity substantially higher than that of Nd₂CuO₄, but no indication of superconductivity. After firing in N₂ the slope is reduced and there is a clear superconducting transition. The onset temperature for superconductivity was found to increase with nominal fluorine content to a maximum of 27 K for $x=0.3$, and then to decrease, reaching 10 K in an $x=0.5$ sample, which was no longer single-phase. No superconductivity was observed in a sample of nominal composition $x=0.1$. The superconducting transitions were broad in all cases and depended on the details of the sample composition and preparation. The sharpest transition observed was in a sample of Nd₂CuO_{3.6}F_{0.4}; in this case the superconducting onset temperature was 21 K and zero resistance was observed at 12 K. Flux-exclusion measurements on two samples with $x=0.2$ and 0.4 each showed 13% Meissner effects. All of the fluorinated samples exhibited n-type conductivity at room temperature, as determined by Seebeck-effect measurements. The fluorinated Pr copper oxides Pr₂CuO_{4-x}F_x ($x > 0.2$) also super-

conduct; the a.c. susceptibility exhibits a strong diamagnetic signal at 4.2 K and the resistivity shows a sharp drop at temperatures as high as 13 K (for the $x=0.4$ sample). However, zero resistance at 4.2 K was not achieved in any of the Pr samples. Although samples containing rare earths other than Pr could not be made as a single phase for the reasons mentioned above, screening of multiphase materials by a.c. susceptibility has failed to show superconductivity.

Although F and O are similar in size, their mutual substitution is often limited, especially in transition-metal compounds. The extent of substitution of fluorine in YBa₂Cu₃O₇ is limited, and its effect on superconductivity is detrimental⁴, because YBa₂Cu₃O₇ is a p-type conductor in the normal state so that fluorine doping reduces the carrier density. That substantial fluorine substitution is possible in Nd₂CuO_{4-x}F_x may be explained on the basis of its T' structure⁵ (Fig. 2). The structure contains two oxygen atom sites: O(1) is bonded to Cu, forming the Cu-O square net common to all copper oxide superconductors, whereas O(2) is bonded to Nd, forming a fluorite-type layer between the CuO₂ sheets. It is the O(2) atoms that we believe to be substituted by F. Substitution on this site would not disrupt the CuO₂ plane and would make the bonding of the NdO_{2-x}F_x layer similar to that of NdOF, which has a distorted fluorite structure.

The phase purity and substantial flux exclusion below T_c of the T'-phase Nd₂CuO_{4-x}F_x indicate bulk superconductivity, but the broad superconducting transitions and semiconducting slope of the electrical conductivity above T_c suggest that the samples are not completely homogeneous. Similar behaviour has been observed in ceramic samples of other solid-solution superconductors La_{1.85}Sr_{0.15}CuO₄ (ref. 6) and Pb₂Sr₂Y_{1-y}Ca_yCu₃O₈ (ref. 7). □

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1. Tokura, T., Takagi, H. & Uchida, S. *Nature* **337**, 345-347 (1989).
2. Batsanova, L. R. & Podberezskaya, N. V. *Russ. J. Inorg. Chem.* **11**, 534-535 (1966).
3. Manthiram, A., Swinnea, J. S., Sui, Z. T., Steinfink, H. & Goodenough, J. B. *J. Am. chem. Soc.* **109**, 6667-6669 (1987).
4. Zahurak, S. M. et al. *Solid State Ionics* (in the press).
5. Müller-Buschbaum, H. & Wollschläger, W. *Z. anorg. allg. Chem.* **414**, 76-80 (1975).
6. Van Dover, R. B., Cava, R. J., Batlogg, B. & Rietman, E. A. *Phys. Rev. B* **35**, 5337-5339 (1987).
7. Cava, R. J. et al. *Nature* **336**, 211-214 (1988).

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Optical study of the metal-insulator transition on Ba_{1-x}K_xBiO₃ thin films

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THE superconducting perovskite (Ba, K)BiO₃ (refs 1, 2) has the highest transition temperature known ($T_c \approx 31$ K) aside from the copper oxide compounds³. This system becomes superconducting upon the substitution of monovalent potassium for divalent barium in the parent insulator BaBiO₃, analogous to the substitution of strontium for lanthanum in La₂CuO₄. To understand whether the mechanism of the superconductivity is the same as in the copper oxide compounds, it is important to know the origin of the insulating state of the parent material, as well as the basic character of the metallic state induced by the cation substitution. In the case of its predecessor Ba(Bi,Pb)O₃, optical measurements were used to investigate the change in electronic state across the metal-insulator transition at 65% lead doping⁴. Here we report the successful synthesis of (Ba,K)BiO₃ thin films, which has allowed

us to measure the optical spectrum, as well as the transport coefficients of $(\text{Ba},\text{K})\text{BiO}_3$. Our results, combined with the previous report of the X-ray crystal structure⁵, strongly suggest that the Peierls gap, responsible for the insulating state of BaBiO_3 , almost completely vanishes at the metal-insulator transition, resulting in a metallic state described by band theory⁶.

The $\text{Ba}_{1-x}\text{K}_x\text{BiO}_3$ thin films were epitaxially grown on SrTiO_3 (110) substrates using an r.f.-magnetron sputtering method with substrate temperature of 400 °C. The sputtering targets were prepared from a powder mixture of Bi_2O_3 , BaO and KO_2 by means of the two-step synthesis procedure⁷. The composition of the targets was adjusted so as to reduce the deviation of the composition of the sputtered films from the stoichiometric ratios, because films sputtered from a stoichiometric target contained a large number of K and Bi vacancies. The chemical composition of the films was determined roughly by electron-probe microanalysis and X-ray fluorescence analysis. In particular, the K composition (x) was estimated from the lattice constant⁸. The K composition was sensitive to both the argon gas pressure and the oxygen partial pressure. The superconducting films with the highest potassium concentration, $x=0.39$, were obtained by using a gas mixture of argon with 1% oxygen at a pressure of $\sim 10^{-2}$ torr. Under these conditions, the deposition rate was $\sim 2 \text{ \AA s}^{-1}$ and the typical thickness of the films was $\sim 3,000 \text{ \AA}$. Because of the reducing growth conditions, the films had large numbers of oxygen vacancies; these were filled by annealing the films in flowing oxygen at $\sim 500 \text{ °C}$ for 1 h.

Figure 1 shows the X-ray diffraction pattern; the films are almost perfectly orientated in the (110) direction without any trace of a secondary phase. The width of the diffraction peak is comparable to that of the single-crystalline substrate, which demonstrates the good quality of the films.

Not all of the films were superconducting. After oxygen annealing, the films with $x > 0.35$ had a very sharp superconducting transition, whereas films with $x < 0.35$ remained semiconducting. The superconducting transition was observed with both resistivity and magnetization measurements. As seen from the inset of Fig. 2, the onset transition temperature is 23 K, the transition width being no more than 0.2 K. The sharp transition and the high critical-current density at 4.2 K of $7 \times 10^4 \text{ A cm}^{-2}$ (which is comparable with the value of $2.5 \times 10^5 \text{ A cm}^{-2}$ reported for a $\text{Ba}(\text{Bi},\text{Pb})\text{O}_3$ single-crystalline thin film⁹) testify to the homogeneity of the films.

From 300 to 150 K, the resistivity is almost constant and is $< 1 \text{ m}\Omega \text{ cm}$, indicating that grain boundaries have a smaller effect

than in the polycrystalline specimens, for which significantly higher resistivity has been reported^{10,11}. Below 150 K the resistivity has a positive (that is, metallic) temperature coefficient, which has never been observed for polycrystalline samples.

The metallic nature is also confirmed by the optical reflectivity spectrum. Figure 2 shows a reflectivity edge due to free-carrier plasma for $\text{Ba}_{0.63}\text{K}_{0.37}\text{BiO}_3$ thin films. This reflectivity spectrum fits the Drude free-electron model well in the energy range from 0.5 to 2.0 eV. The fit is not so good in the energy region above 2 eV because of the presence of an absorption band at $\sim 3 \text{ eV}$. The fitting parameters are $\hbar\omega_p = 1.59 \text{ eV}$, $\hbar\gamma = 0.92 \text{ eV}$ and $\epsilon_\infty = 3$, where ω_p , γ and ϵ_∞ are the plasma frequency, the damping factor and the optical dielectric constant, respectively. The 'bare' plasma energy $\epsilon_\infty^{1/2}\hbar\omega_p$ (formally equal to $(4\pi ne^2/m^*)^{1/2}$, m^* and n being the effective mass and the density of carriers, respectively) is 2.8 eV, which is $\sim 50\%$ smaller than the value of 4.1 eV given by band calculations¹². However, in view of the uncertainty in the value of ϵ_∞ due to the absence of higher-energy reflectivity data, the agreement between experimental results and band theory calculations is satisfactory. The estimated conductivity at zero energy, $\sigma_{\text{d.c.}} = \epsilon_\infty\omega_p^2/\gamma$, obtained from these parameters is $\sim 2 \times 10^3 \Omega^{-1} \text{ cm}^{-1}$ and is nearly consistent with the resistivity measurement.

The spectrum is significantly different from that of superconducting $\text{BaBi}_{0.25}\text{Pb}_{0.75}\text{O}_3$, for which $T_c = 12 \text{ K}$. In the latter case, the plasma spectrum does not fit the Drude model even in the energy region below 1 eV owing to the specific circumstances described below⁴.

Taking advantage of the synthesis of these thin films, we investigated the metal-insulator transition using transmittance measurements. Figure 3a shows the optical-conductivity spectra obtained from both reflectivity and transmission measurements on BaBiO_3 films and those with K substituted at $x = 0.17, 0.28$ and 0.37. For comparison, Fig. 3b shows the optical-conductivity spectra of $\text{BaBi}_{1-x}\text{Pb}_x\text{O}_3$, which were obtained from the reflectivity spectra by means of a Kramers-Kronig transformation⁴.

A rather sharp dominant absorption peak is observed at 2 eV in the spectrum of BaBiO_3 , the parent compound of both systems, which we therefore interpreted to be a Peierls insulator with an optical energy gap of $\sim 2 \text{ eV}$ (ref. 4), similar to the simple cubic metals originally considered by Peierls¹³. In BaBiO_3 , the

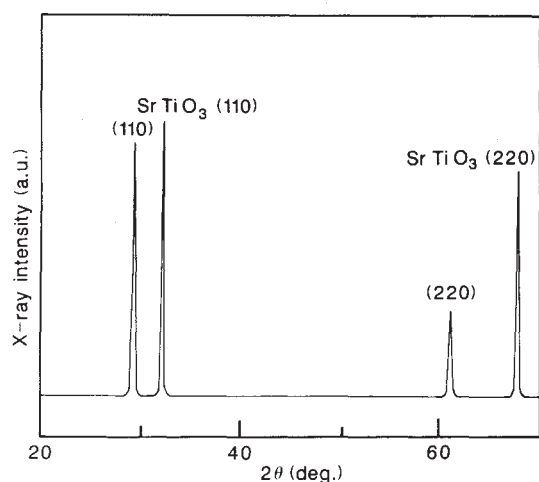


FIG. 1 $\text{Cu K}\alpha_1$ X-ray diffraction pattern of a 3,000-Å-thick film of $\text{Ba}_{1-x}\text{K}_x\text{BiO}_3$ on a SrTiO_3 (110) substrate. The calculated lattice parameter was 4.275 Å, which corresponds to a K concentration $x=0.39$, according to ref. 8. X-ray intensity is expressed in arbitrary units.

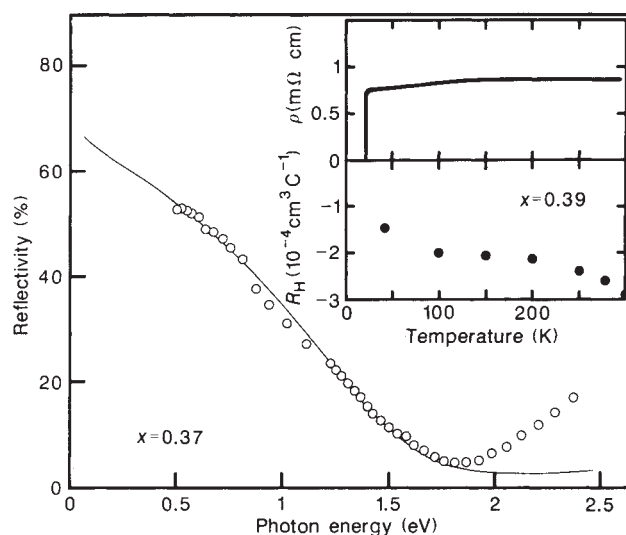
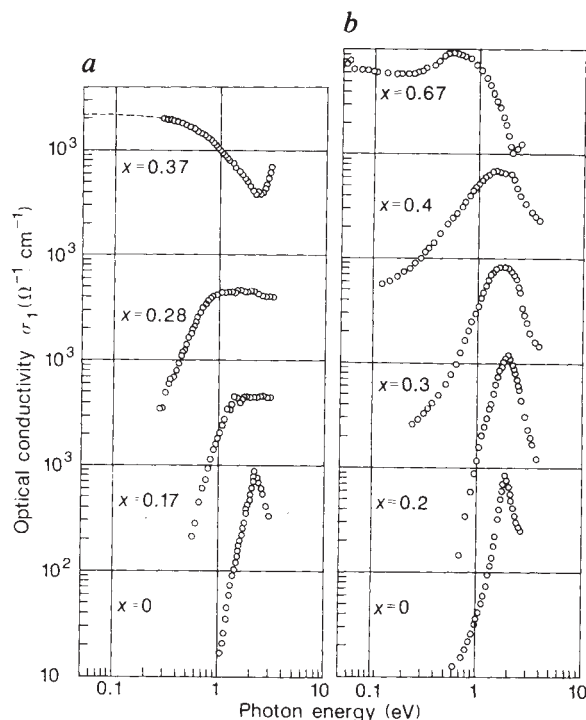


FIG. 2 The reflectivity spectrum for a 1,000-Å-thick film with $x=0.37$ in the energy region from 0.5 to 2.4 eV. The solid line shows the spectrum calculated based on the Drude model with the plasma frequency $\hbar\omega_p = 1.59 \text{ eV}$, the damping frequency $\hbar\gamma = 0.92 \text{ eV}$ and the optical dielectric constant $\epsilon_\infty = 3$. The inset shows the temperature dependence of the electrical resistivity, ρ , and the Hall coefficient, R_H , of a thin film with $x=0.39$ and a thickness of 3,000 Å.

FIG. 3 *a*, Optical-conductivity spectra for $\text{Ba}_{1-x}\text{K}_x\text{BiO}_3$ thin films with $x=0$ (BaBiO_3), 0.17, 0.28 and 0.37. The thickness of the films were 3,000, 20,000, 10,000 and 1,000 Å, respectively. The broken line in the spectrum for the film with $x=0.37$ denotes the calculated spectrum below 0.25 eV using the Drude model with the parameters stated in the text. *b*, Optical-conductivity spectra for the $\text{BaBi}_{1-x}\text{Pb}_x\text{O}_3$ system obtained from the reflectivity spectra by means of a Kramers-Kronig transformation.



Bi atoms occupy the sites of a simple cubic lattice, but the oxygen octahedra surrounding Bi sites are alternately expanded and compressed (breathing-mode distortion), as observed in neutron-scattering experiments¹⁴. Such periodic lattice distortion leads to Bi^{3+} - Bi^{5+} charge ordering or charge disproportionation ($\text{Bi}^{4+} + \text{Bi}^{4+} \rightarrow \text{Bi}^{3+} + \text{Bi}^{5+}$), giving rise to so-called charge density waves (CDWs). As a consequence of CDW formation, an energy gap (Peierls gap) opens at the centre of the conduction band composed of $\text{Bi}(6s)$ - $\text{O}(2p)$ antibonding orbitals¹⁵.

When Bi is partially substituted by Pb, the absorption peak is gradually broadened and shifts towards a lower energy with a tail extending to much lower energies. This has been attributed¹⁶ to the formation of Pb states within the Peierls gap of BaBiO_3 . The metal-insulator transition is considered to take place at 65% Pb substitution, when the $\text{Pb}(6s)$ states fill up the energy gap. In this picture, the Peierls gap persists as a 'pseudogap' even in the metallic phase⁴. This is supported by the fact that the optical-conductivity spectrum for the metallic compound $\text{BaBi}_{0.33}\text{Pb}_{0.67}\text{O}_3$ retains the absorption peak.

The case of K doping is somewhat different. As in the case of Pb substitution, the absorption edge shifts towards lower energy with increasing K, clearly demonstrating a decrease in the optical energy gap. However, the optical conductivity still rises steeply without any appreciable low-energy tail and the edge shifts faster than in Pb-substituted compounds. Single-crystal X-ray analysis for compounds with little K substitution⁵ shows two distinct Bi sites generated by a kind of breathing-mode lattice distortion. The optical spectrum suggests that the K-substituted compound is still a Peierls insulator but that the energy gap is reduced more rapidly by K-substitution. This is consistent with the fact that, in the $\text{Ba}_{1-x}\text{K}_x\text{BiO}_3$ system, the critical composition at which the metal-insulator transition takes place is $x=0.35$, whereas it does so for $x=0.65$ in the $\text{BaBi}_{1-x}\text{Pb}_x\text{O}_3$ system. The difference between the two systems arises from the difference in the electrostatic potential produced by the different substitutions in the parent compound BaBiO_3 . It is inferred from the optical spectra that substituted Pb atoms give rise to energy states within the energy gap whereas K atoms do not.

The optical conductivity spectrum of the metallic compound with $x=0.37$ is featureless below the plasma energy, suggesting that the energy gap would vanish almost completely at the

insulator-metal transition, in contrast to the case of $\text{Ba}(\text{Bi,Pb})\text{O}_3$ where the gap remains even in the metallic phase. This might be the reason for a lower T_c value ($T_c = 12$ K) in this compound.

Measurements of the Hall and Seebeck effects on metallic thin films give negative coefficients. The Hall coefficient, shown in the inset of Fig. 2, is $-3.0 \times 10^{-4} \text{ cm}^3 \text{ C}^{-1}$ at 300 K and the Seebeck coefficient is $-(1-2) \mu\text{V K}^{-1}$ in the temperature range 80–350 K. That the charge carriers are electrons is plausible if the energy gap vanishes and the resultant single conduction band is less than half-full as a result of K doping. Single-crystal X-ray diffraction⁵ indicates that Bi sites remain equivalent, so there is no lattice distortion in the superconducting phase of $\text{Ba}_{1-x}\text{K}_x\text{BiO}_3$. In this respect the normal state of metallic $\text{Ba}_{1-x}\text{K}_x\text{BiO}_3$ seems completely different from that in copper oxide superconductors, in which the energy gap generated by the large Coulomb repulsive energy is preserved and the substitution of divalent for trivalent elements, for example Sr for La in La_2CuO_4 , produces a hole in the lower split-off band.

The optical measurements on thin films of the $\text{Ba}_{1-x}\text{K}_x\text{BiO}_3$ system strongly suggest that K doping reduces the Peierls condensation energy in BaBiO_3 much more effectively than does Pb doping, and the metal-insulator transition in this system is directly related to the destruction of the Peierls gap, whereas in the $\text{BaBi}_{1-x}\text{Pb}_x\text{O}_3$ system the transition is driven by the growth of the Pb states within the Peierls gap. □

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- Mattheiss, L. F., Gyorgy, E. M. & Johnson, D. W. Jr *Phys. Rev.* **B37**, 3745–3746 (1988).
- Cava, R. J. *et al. Nature* **332**, 814–816 (1988).
- Bednorz, J. G. & Müller, K. A. *Z. Phys.* **B64**, 189–193 (1986).
- Tajima, S. *et al. Phys. Rev.* **B32**, 6302–6311 (1985).
- Schneemeyer, L. F. *et al. Nature* **335**, 421–423 (1988).
- Mattheiss, L. F. & Hamann, D. R. *Phys. Rev. Lett.* **60**, 2681–2683 (1988).
- Hinks, D. G. *et al. Physica C* **156**, 477–480 (1988).
- Hinks, D. G. *et al. Nature* **333**, 836–838 (1988).
- Suzuki, M. & Murakami, T. *J. appl. Phys.* **56**, 2330–2331 (1984).
- Welp, U. *et al. Physica C* **156**, 27–34 (1988).
- Kondoh, S., Sera, M., Fukuda, K., Ando, Y. & Sato, M. *Solid St. Commun.* **67**, 879–880 (1988).
- Mattheiss, L. F. & Hamann, D. R. *J. appl. Phys.* **24**, 6–9 (1985).
- Peierls, R. E. *Quantum Theory of Solids*, 112–114 (Clarendon Press, Oxford, 1955).
- Challott, C. *et al. Solid St. Commun.* **56**, 829–830 (1985).
- Mattheiss, L. F. & Hamann, D. R. *Phys. Rev.* **B28**, 4227–4241 (1983).
- Yoshioka, D. & Fukuyama, H. *J. phys. Soc. Jap.* **54**, 2996–3000 (1985).

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Changes of tropical sea-air interaction processes over a 30-year period

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RECENT investigations¹⁻⁵ including radiosonde data from Southern India⁶ have shown that the average tropospheric temperature in the tropics has increased since 1965 by nearly 1 °C. More importantly, the water vapour content of the middle troposphere has increased, above the equatorial Pacific, by 20–30%^{4,5}. Here we report investigations on sea-air interaction processes along the main shipping routes of equatorial oceans—the only areas where there have been enough observations (Table 1) to provide a representative time series. The period 1949–79 was selected because of the general lack of bias in the air-sea parameters with the exception of wind speed. The results indicate an intensification of the hydrological cycle, especially over the warmest oceans and they also suggest tropical circulation changes.

The equatorial belt (10° S–10° N) covers an area of 88×10^6 km², with 77% being ocean. Along 14 sections of the busiest shipping routes (Fig. 1), about 2.4 million observations were selected from the trimmed COADS data⁷, covering an area of nearly 8×10^6 km². We also intended to check the spurious increase⁸ of wind speed. We evaluated the sea surface temperature (SST); the sea-air temperature difference (SST–AT); the saturation deficit ($q_s - q_a$, where q is the specific humidity at the sea surface, subscript s denotes saturation at the sea surface, subscript a , saturation in the air), and the scalar wind speed, v_{scal} . Preliminary results⁹ indicate high spatial variability of all sea-air parameters. This was true even on adjacent sections crossing the Equator where all observations had been made on board the same ships with the same equipment (Table 1) which suggests that the wind speed data show real changes as well as the effect of some biases⁸. The saturation deficit ($q_s - q_a$) increases nearly everywhere, with varying intensity; similarly the sea-air temperature difference increases but with regional and seasonal variations. The cross-equatorial changes are particularly large along routes 3–4 and 9–10, where the southern

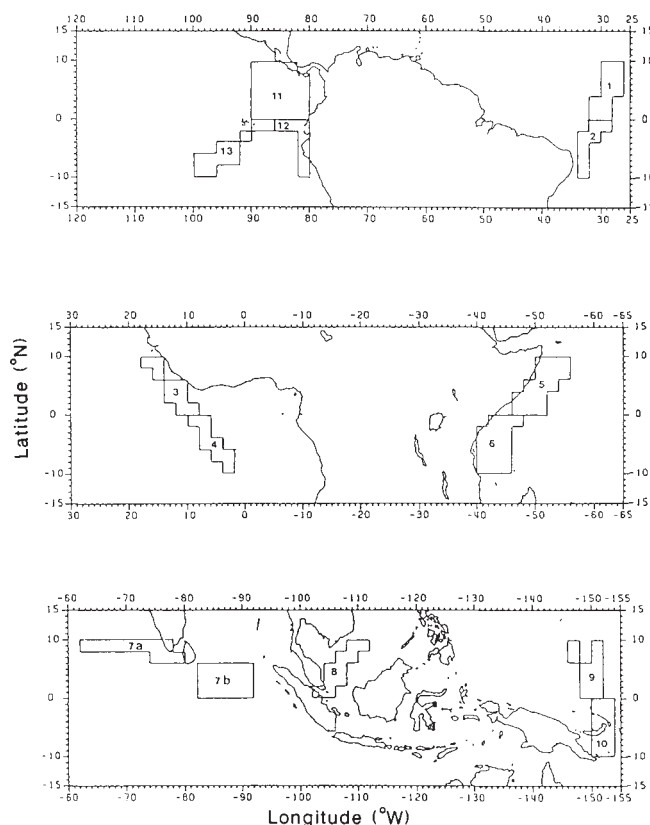


FIG. 1 Map of the selected 14 sections of ship routes in the equatorial belt⁹.

sections are affected by seasonal upwelling.

Table 2 gives 31-yr averages and trends from three regional groups: the Atlantic (sections 1–4); the warmest oceans (60°–180° E, sections 7a, b–10); and the eastern Pacific (11–13). The zonal average (14 sections) is not fully representative, because many routes are in areas with cool water upwelling. For comparison, SST averages from our sections are supplemented by true averages (SST*), using all $4 \times 4^\circ$ COADS fields (J. O. Fletcher, personal communication). The most interesting results are from the warmest oceans on both flanks of Indonesia. In 11 of 14 sections the SST–AT trend is positive, with 7 significant

TABLE 1 Comparison of trends of sea-air parameters (unit per 31 yr) along cross-equatorial section pairs

Sections	Atlantic				Pacific	
	1	2	3	4	9*	10*
SST (°C) Year	(+0.07)	<u>+0.46</u>	(+0.15)	(+0.32)	<u>+0.87</u>	(+0.16)
DJFM†	(+0.04)	+0.41	(+0.01)	(+0.27)	<u>+1.07</u>	(+0.44)
JJAS‡	(+0.13)	<u>+0.45</u>	(+0.30)	(+0.38)	+0.80	(+0.27)
SST–AT (°C) Year	(+0.10)	<u>+0.24</u>	(+0.03)	+0.16	(+0.18)	(–0.05)
DJFM†	+0.13	<u>+0.34</u>	(+0.00)	(+0.08)	(+0.28)	(+0.24)
JJAS‡	(+0.11)	(+0.16)	(+0.03)	<u>+0.24</u>	(+0.01)	(–0.10)
$q_s - q_a$ (g kg ^{–1}) Year	(+0.19)	(+0.21)	(+0.13)	(+0.29)	(+1.02)	(+0.16)
DJFM†	(+0.17)	(+0.20)	(+0.01)	(+0.17)	(+1.26)	(+0.80)
JJAS‡	(+0.27)	+0.28	(+0.14)	(+0.28)	(+0.84)	(–0.17)
v_{scal} (m s ^{–1}) Year	<u>+0.57</u>	(+0.15)	+0.27	<u>+0.63</u>	<u>+1.19</u>	<u>+1.31</u>
DJFM†	<u>+0.69</u>	(+0.27)	(+0.11)	+0.45	(+0.83)	(+0.66)
JJAS‡	(+0.25)	(+0.13)	+0.34	<u>0.82</u>	<u>+1.21</u>	+1.87
Number of observations per month	470	359	769	604	275	258

*Observed 1956–1979 only, corrected to 31 yr.

† DJFM, December January February March.

‡ JJAS, June July August September.

— Significance >99.9%.

– Significance >99%.

() Significance <95%.

at the 95% level. The saturation-deficit trend is significantly positive in sections 6–9; here the 31-yr trend amounts to $+0.72 \text{ g kg}^{-1}$ or $+14\%$. Including wind speed, these values (Table 2) are physically coherent; the strongest positive trends of SST and $(q_s - q_a)$ (Fig. 2) are observed in the warmest areas. These parameters indicate a destabilization of the marine boundary layer, which cannot be systematic error (for instance, the increasing size of ships). The individual points on Fig. 2 suggest that the trends are not affected by the frequency of cold or warm El Niño–Southern Oscillation (ENSO) events.

Recent satellite measurements¹⁰ show high correlations between $(q_s - q_a)$ and evaporation (E); and weaker correlations between v_{scal} and E . This coincides with the increasing moisture in the midtroposphere (500–700 hPa, 1 hPa = 1 mbar) above the Pacific, as discussed in refs 4, 5. Here precipitable water has increased at four stations (each significant at 95%), by up to 30% from 1965 to 1984.

The simultaneous increase of SST and atmospheric moisture can be understood following recent investigations^{11,12} which have revealed a nonlinear increase in the heights of convective

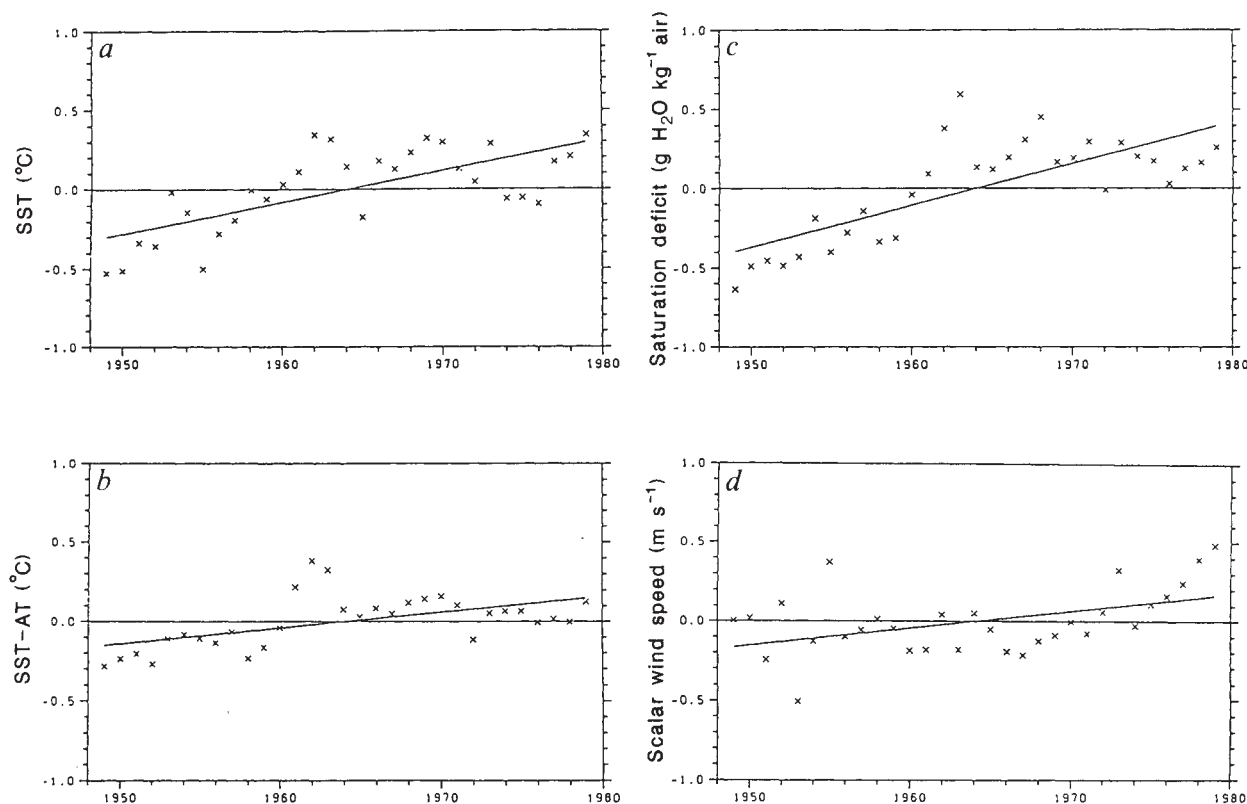


FIG. 2 Time series of selected parameters at the sea-air interface, averaged for sections 7a, 7b, 8–10 (warmest oceans) with linear annual trend 1949–79.

a, SST; b, SST – AT; c, saturation deficit $q_s - q_a$; d, scalar wind speed, v_{scal} .

TABLE 2 Annual averages for equatorial oceans and Indian Ocean seasonal variation

Annual averages for equatorial oceans (10° N–10° S)						
Sections		SST (°C)	SST – AT (°C)	$q_s - q_a$ (g kg ⁻¹)	V_{scal} (m s ⁻¹)	SST* (°C)
Atlantic (Sections 1–4)	<i>M</i>	26.58	0.48	4.72	5.07	26.79
	T_{31}	(+0.25)	+0.13	(+0.20)	+0.41	
East Pacific (Sections 11–13)	<i>M</i>	24.36	0.60	3.70	4.84	26.28
	T_{31}	(+0.14)	–0.13	(–0.02)	+0.52	
Warmest Oceans (Sections 7a, 7b–10)	<i>M</i>	28.55	0.58	5.50	4.85	28.50
	T_{31}	+0.39	+0.16	+0.55	+0.74	
Zonal averages (14 Sections)	<i>M</i>	26.87	0.50	4.76	5.10	27.39
	T_{31}	+0.34	+0.12	+0.33	+0.53	
Seasonal variation in the equatorial Indian Ocean (0–10° N, sections 5, 7a + 7b)						
Summer (JJAS)	<i>M</i>	27.21	+0.02	4.00	7.73	
	T_{31}	+0.46	+0.31	+0.57	(–0.01)	
Winter (DJFM)	<i>M</i>	27.69	0.40	5.51	5.00	
	T_{31}	+0.45	+0.22	+0.37	+0.41	

SST*, 'true' average from complete data ($4^\circ \times 4^\circ$ fields, COADS). *M*, annual averages for the period 1949–79. T_{31} , Trend (unit = *M*). Significance symbols as in Table 1.

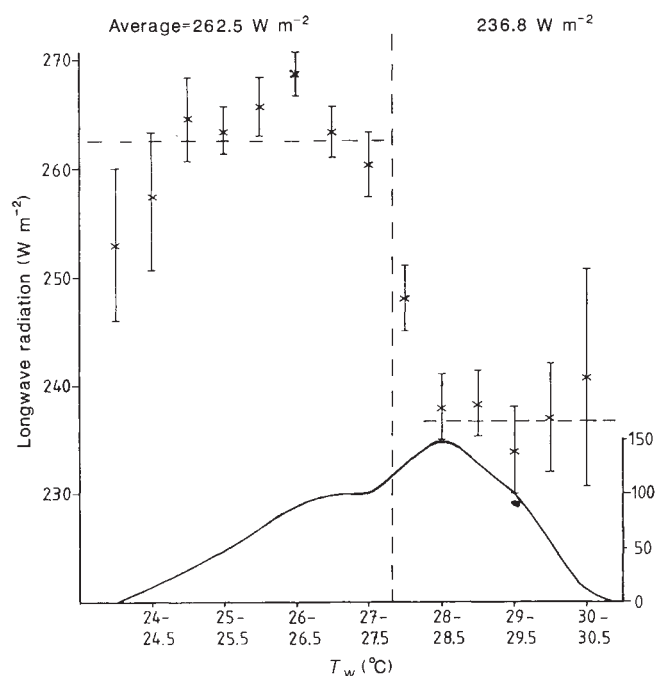


FIG. 3 Outgoing longwave radiation—representing cloud-top temperature and height—as a function of SST (T_w) at the surface of the equatorial Pacific Ocean¹¹. Lower curve denotes frequency of cases.

cloud tops with increases in SST. The statistics (Fig. 3) show a jump between high, cold and low, warm cloud tops near SST = 27.5°C. Strong convective activity driven by low-level convergent winds¹² transports water vapour to the upper troposphere, where latent heat is released by condensation. An evaluation from COADS-based data yields an ocean area of $\sim 65 \times 10^6 \text{ km}^2$ with SST > 27.5°C. With a warming of 0.39°C over 31 years (Table 2) this area has grown, over this period, by $\sim 13 \times 10^6 \text{ km}^2$ or 20%. Such figures seem to hold the key for understanding such rapid development even assuming no variation of the wind field.

These changes of sea-air parameters are correlated with an increase in E . Disregarding absolute values of E , we may estimate, on the basis of the well known bulk formula¹³ the relative changes between 1949 and 1979. Assuming no change of wind speed (which is unlikely), we obtain from the $(q_s - q_a)$ trend alone an increase of E over the 'warmest' oceans of $\sim 10\%$; if we allow an increase in v_{scal} of 0.25 m s^{-1} , the rise in E amounts to $\sim 15\%$ in 31 years. As the 1927 Beaufort wind scale used in COADS data has to be shifted upward (by $1.4\text{--}2.0 \text{ m s}^{-1}$ after comparison with measurements in different latitudes^{13–15}), it is necessary to add a correction of $\sim +1.5 \text{ m s}^{-1}$ to the average COADS wind speeds. Then the additional energy input from release of latent heat into the atmosphere is $10\text{--}15 \text{ W m}^{-2}$. This should be compared with a global value of $2\text{--}2.5 \text{ W m}^{-2}$ from the increase in CO_2 and other greenhouse gases¹⁶, not accounting for feedback mechanisms, since the beginning of this century. Similar ratios have been estimated before^{17–19}.

Independently two recent climate models have indicated a heating maximum in the tropics^{19,20}. Recent model studies have also found that positive feedback coefficients operating by means of water vapour and clouds (primarily in the tropics) are distinctly higher than the snow-albedo feedback²¹.

This recently discovered powerful heating source in the equatorial belt is concentrated over the eastern Indian Ocean and Western Pacific. Here the upper mixed layer of the ocean is effectively isolated from exchange with the cool layers below, owing to the great depth of the mixing layer and the lack of effective upwelling. The rise of SST, of $(q_s - q_a)$ and of E together

with the increase in tropospheric temperature and moisture⁴ are consistent with a strengthening of the subtropical 700-hPa westerlies in the Pacific section²². This suggests an intensification of the Hadley circulation in that section and has been confirmed by our investigations at 200- and 500-hPa levels (to be reported elsewhere).

If we consider the zonal SST differences between the warmest oceans and sections 11–13 as representing the Walker circulation, our data (Table 2, upper panel) show an increase of this SST difference of 6%; no radiosonde time series are available at the Equator. Land data indicate, for 1950–80, no significant trend²³ at latitudes 35–60° N. In the Indian Ocean, therefore, the rise of SST and of the heating rate (indicated by SST – AT) at sections 5, 7a and 7b (Table 2, lower panel) suggest a decrease (increase) of the meridional land–sea difference during summer (winter) that drives both monsoons in these sections. Taking into account (as above) an estimated real increase of surface wind speed $+0.25 \text{ m s}^{-1}$, the wind data reveal a weakening of the summer monsoon and an intensification of the winter monsoon.

It seems that the warming of the tropical troposphere can be associated with more frequent deep convection correlated with SST > 27.5°C releasing more latent heat into the atmosphere. The physical role of water vapour is not only due to its (radiative) greenhouse effect, but even more to its phase changes^{16,18}. This provides an additional energy input to the atmosphere. The resulting positive feedback leads to an acceleration of the delayed ocean warming in low latitudes^{19,20}, to a strengthening of most tropical circulations and perhaps also to a greater frequency and/or intensity of warm ENSO events.

Because we have only short observation periods, we refrain from extrapolating these trends into the future. However, the search for a warming signal should be directed primarily at the tropics, instead of higher latitudes.

In the upwelling regions of the tropical oceans, the sea–air fluxes of CO_2 and H_2O are nearly simultaneously intensifying and weakening²⁴, during ENSO cycles. Among other considerations, this also suggests that the manmade increase in the atmospheric CO_2 concentration could be the primary trigger of a chain of processes within the tropical branch of the climate system. At present, we do not wish to speculate about the possible consequences of such an increase of the hydrological cycle in a key area of the climate system. We wish to bring the observed signal to the attention of others concerned with global warming. □

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- Parker, D. E. *J. Climatol.* **5**, 273–282 (1985).
- Weber, K.-H. *et al. Ann. Met. N.F.* **23**, 5–6 (1986).
- Barnett, T. P. *J. geophys. Res.* **91**, 6659–6667 (1986).
- Hense, A. *et al. Met. atmos. Phys.* **38**, 215–227 (1988).
- Krahe, P. *et al. Tropical Ocean–Atmosphere Newsl.* **38**, 11–13 (1987).
- Rupa Kumar, K. *et al. J. Clim. appl. Met.* **26**, 304–314 (1987).
- Woodruff, S. D. *et al. Bull. Am. Met. Soc.* **68**, 1239–1250 (1987).
- Ramage, C. S. *J. Clim. appl. Met.* **26**, 525–528 (1987).
- Flohn, H. & Kapala, A. *Tropical Ocean–Atmosphere Newsl.* **45**, 4–7 (1988).
- Liu, W. T. *J. geophys. Res.* **93**, 6749–6760 (1988).
- Gadgil, S. *et al. Nature* **312**, 141–143 (1984).
- Graham, N. E. & Barnett, T. P. *Science* **238**, 657–659 (1987).
- Isemer, H. D. & Hasse, L. *The Bunker Climatic Atlas of the North Atlantic Ocean Vol. 2* (Springer, Berlin, 1987).
- Kuhlbrodt, L. *Ann. Hydrogr. mar. Met.* **64**, Köppen Heft 14–23 (1936).
- Kaufeld, L. *Meteor. Rundsch.* **34**, 17–23 (1981).
- Ramanathan, V. *Science* **240**, 293–299 (1988).
- Möller, F. *J. geophys. Res.* **68**, 3877–3886 (1963).
- Ramanathan, V. *J. Atmos. Sci.* **38**, 918–930 (1981).
- Wilson, C. A. & Mitchell, J. F. B. *J. geophys. Res.* **92**, 315–318, 343 (1987).
- Hansen, J. *et al. J. geophys. Res.* **93**, 9341–9346 (1988).
- Schlesinger, M. E. *Clim. Dyn.* **1**, 35–50 (1986).
- Namias, J. *et al. J. Clim.* **1**, 682–703 (1988).
- Editors, *World Climate Data Programme (WCP): Climate System Monitoring (CSM) Mon. Bull. No. 1987–3*.
- Weber, K.-H. & Flohn, H. *Bonn. Met. Abhandl.* **31**, 73–107 (1984).

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Impact production of CO₂ by the Cretaceous/Tertiary extinction bolide and the resultant heating of the Earth

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EVIDENCE at the Cretaceous/Tertiary boundary suggests that the proposed 'extinction' bolide¹ struck a continental or shallow marine terrane. This evidence includes: shocked quartz and feldspar grains found in the boundary layer inherited from a range of rock types^{2,3}; a high ⁸⁷Sr/⁸⁶Sr ratio in some planktonic fossils⁴ which could reflect continental-derived Sr (ref. 5); and evidence that the platinum-group-element-rich clay layer is underlain (at some localities) by a deposit of possible tsunamic origin^{6,7}. These observations and data demonstrate that sea level at the end of the Cretaceous was ~150–200 m higher than at present, suggesting the possibility that the extinction bolide struck a shallow marine carbonate-rich sedimentary section. Here we show that the impact of such a bolide (~5 km in radius) onto a carbonate-rich terrane would increase the CO₂ content of the atmosphere by a factor of two to ten. Additional dissolution of CO₂ from the ocean's photic zone could release much larger quantities of CO₂. The impact-induced release of CO₂, by itself, would enhance atmospheric greenhouse heating and give rise to a worldwide increase in temperature from 2 K to 10 K for periods of 10⁴ to 10⁵ years.

Many impact-induced phenomena have been suggested to exert a climatic effect. Alvarez *et al.*¹ proposed that the shielding of the Sun by dust injected into the atmosphere following a land impact would exert such an influence on global climate; this was later explored by others^{8–10}. There is now good evidence that large amounts of soot, possibly from shock-induced forest fires, occur in the Cretaceous/Tertiary (K/T) layer¹¹. An impact into the ocean can also induce a short-lived (<0.1 year) greenhouse effect caused by water vapour¹². The impact-driven atmospheric shock wave would produce large quantities of NO_x (refs 13–15), which could deplete the ozone layer through photo-

chemical processes. Acidification of the atmosphere and land and ocean surfaces is also expected¹⁶, which would result in increased atmospheric CO₂ upon dissolution from the ocean surface. Enhanced absorption of solar radiation by NO_x and CO₂ can affect the atmospheric thermal-radiation balance, and hence global climate.

The amount of CO₂ released by impact-induced shock pressures in carbonate minerals can be estimated using the experimental results of Lange and Ahrens¹⁷ and the impact scaling relations of O'Keefe and Ahrens¹⁸. Lange and Ahrens performed a series of laboratory impact experiments in which they measured the amount of CO₂ released as a function of peak shock pressure (Fig. 1). They found that the threshold shock pressure required to induce devolatilization of CaCO₃ to CaO and CO₂ is ~10 GPa.

The volume of vapour (V_v) relative to the volume of the impactor (V_m) is given by

$$\frac{V_v}{V_m} = 0.132 \left(\frac{\rho_m}{\rho_s} \right) \frac{U^2}{\Delta E} \quad (1)$$

where ρ_m and ρ_s are the densities of the impactor and planetary surface, U is the velocity of impact and ΔE is the energy of vaporization; for silicates, $\Delta E \approx 0.2 \times 10^{12}$ erg g⁻¹ (ref. 18). The effective energy of devolatilization of calcium carbonate was derived by scaling this energy of vaporization by the ratio of the pressures for incipient impact devolatilization:

$$\begin{aligned} \Delta E_{\text{CaCO}_3} &= 0.2 \times 10^{12} P_{\text{CaCO}_3}^v / P_{\text{sil}}^v \\ &= 9.5 \times 10^9 \text{ erg g}^{-1} \end{aligned} \quad (2)$$

where $P_{\text{CaCO}_3}^v$ and P_{sil}^v are the threshold pressures for incipient vaporization of calcite and silicates, respectively. We used this value of ΔE in equation (1) to calculate the volume of CO₂ produced.

The scaling relation given by equation (1) was derived from calculations of impact upon a homogeneous planet. Carbonate-rich sections are expected to range in thickness up to 4 km. For large-scale impacts (impactor radii >5 km) the peak shock pressures are expected to completely devolatilize the carbonate-rich layer. Because the shock impedances of carbonate rocks are similar to those of silicates, the shock trajectories and pressures do not differ significantly between a homogeneous silicate planet and a carbonate sequence overlying a silicate half-space. With this assumption, the volume, V_L , of the carbonate layer that would be devolatilized is

$$V_L = \pi [R_v^2 d - \frac{1}{3} d^3] \quad (3)$$

where R_v is the radius of the region that is shocked to pressures sufficient for devolatilization and d is the mean thickness of the carbonate layer. Here R_v is given by

$$R_v = \left(\frac{6}{4\pi} V_v \right)^{1/3}$$

Our model has two layers: a carbonate-rich layer, which was assumed to be either 1 or 4 km thick, and an underlying silicate rock core. Less than one-half of the Earth's surface, comprising moderate-depth oceanic terrane or continental-shield terrane, is represented by a thin (1-km) carbonate layer, whereas the small fraction of the Earth's surface which has thick sections of shallow marine sediments¹⁹ is represented by a 4-km layer. The total carbonate reservoir of the Earth, 7×10^{22} g, would provide a 400-m-thick layer covering the whole Earth²⁰. We examined the effect of including an ocean with a depth of less than 3 km and concluded that for impactors with radii greater than 1 km, the ocean does not significantly affect the amount of CO₂ released. Our results for the mass of CO₂ evolved as a function of impactor size are shown in Fig. 2. Two types of impactors were considered; asteroids with a density of 3 g cm⁻³ and a velocity of 20 km s⁻¹, and comets with a density of 1 g cm⁻³ and a velocity of 60 km s⁻¹. The mass of CO₂ released exceeds

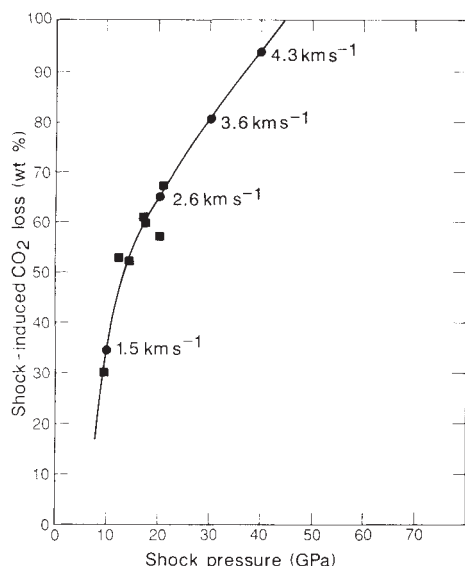


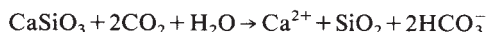
FIG. 1 Fraction of CO₂ released from calcite as a function of impact pressure¹⁷ (squares). The solid line shows theoretical calculations, and corresponding impact velocities are indicated for shock pressures of 10, 20, 30 and 40 GPa (circles).

the present-day atmospheric content of 2.7×10^{18} g when the radius of asteroid impactor is greater than 3.1 and 6 km for a 4- and 1-km-thick carbonate layer respectively, and when the corresponding radius of a comet impactor is greater than 2.1 and 4.2 km. On the basis of the iridium content at the K/T boundary layer, Alvarez *et al.*¹ estimated the minimum radius of the K/T asteroid to be 5 ± 2 km. The radius of a cometary impactor must be 1.4 times that of an asteroidal impactor to give the same effect. For the above range of K/T impactor radii, it is clear that significant amounts of CO_2 can be ejected into the atmosphere.

In the case of impactors whose radii are of the order of a fraction of the atmospheric scale height (~ 7 km), the devolatilized CO_2 would be dispersed rapidly and globally. In contrast, the CO_2 -depleted rock is expected to remain buried in the crater and its recarbonation must be retarded by the overlying ejecta blanket. Roddy *et al.*²¹ and O'Keefe and Ahrens²² have calculated the flow fields in the Earth and atmosphere that would result from the impact of a 5-km-radius bolide causing the K/T event. Although neither calculation included explicitly the effects of CO_2 devolatilization, it can be inferred from the geometry and evolution of the flow fields of the Earth and atmosphere that the evolved CO_2 would be propelled upward from the impact site and be distributed globally.

The sudden injection of CO_2 or water into the atmosphere will increase the Earth's mean surface temperature through the greenhouse effect²³. The time required for the atmosphere to heat up by either mechanism is much less than the radiative cooling time (~ 3 days). The re-equilibration time of the solid-Earth/ocean/atmosphere system to a sudden large increase in CO_2 in the atmosphere is relatively long, whereas the re-equilibration time for an increase in atmospheric water is in the range of months to several years¹².

The re-equilibration of CO_2 by dissolution in the deep ocean water or dissolution of deep-sea sediments may occur on time-scales of 10^3 to 10^4 years (ref. 24). Reaction with crustal rock is thought to occur through the rock-weathering reaction



with Ca^{2+} and HCO_3^- subsequently removed by carbonate precipitation from sea water on a timescale of 10^3 years (ref. 25). Surface sea water is at present supersaturated with respect to bicarbonate ion, and an increase of the ocean surface temperatures, as well as production of NO_x and its subsequent uptake by the oceans, is expected to release additional CO_2 to the atmosphere^{16,26}.

We used the results of Kasting and Ackerman²⁷ to obtain the temperature rise at the Earth's surface as a function of CO_2

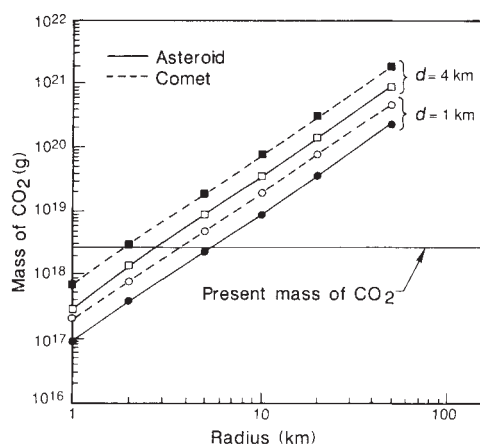


FIG. 2 Mass of CO_2 released during an impact from a carbonate sediment. The impact velocity is 20 km s^{-1} and the density of the impactor is 3 g cm^{-3} for an asteroid and 1 g cm^{-3} for a comet. The carbonate thickness, d , is either 1 or 4 km.

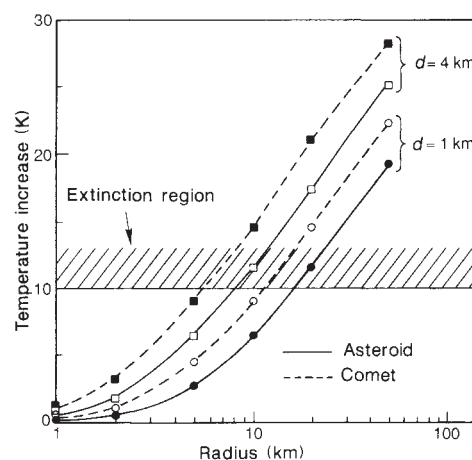


FIG. 3 Mean rise in atmospheric temperature as a function of impactor radius. Impact conditions are described in Fig. 2. The extinction region¹² is indicated.

content and to calculate the temperature rise as a function of the size of the impactor from the conditions shown in Fig. 3. The increase in temperature as a function of mass of CO_2 in the atmosphere is given by $\Delta T = 10 \log_{10} m/m_0$, where m is the mass of CO_2 in the perturbed atmosphere and m_0 is the mass of CO_2 in the normal atmosphere²⁷. The amount of CO_2 obtained in Fig. 2 is conservative, as it comes only from impacted carbonates and includes neither the effect of additional CO_2 expected from surface heating or acidification of the ocean²⁸, nor that induced by biomass destruction. Figure 3 indicates that the temperature change calculated is significant (> 5 K) for impactors greater than 3 km in radius. Emiliani *et al.*¹² have argued that the intolerance of large families of biota to increases in temperature of ~ 10 K would be sufficient to account for the K/T extinctions. Moreover, if such a temperature excursion remained for $> 10^3$ yr, then global oceanic warming would also occur and major and complex changes in oceanic currents would result. This may be reflected in the generally unsteady and poorly correlated values of $\delta^{18}\text{O}$ measured for planktonic foraminifera tests over the first 10^5 yr of the Tertiary^{29,30}. The most compelling evidence for a long period of extended CO_2 -enrichment of the atmosphere, and by inference, an extended global temperature increase, stems from the 10^3 – 10^4 -yr period of ^{13}C -depleted surface foraminifera tests and the concurrent absence of the usual relationship between heavy carbon ($\delta^{13}\text{C} = +3$ at the surface) and light carbon ($\delta^{13}\text{C} = +1$ at depth) measured in the normal biologically active ocean.

Emiliani *et al.*¹² and McLean³¹ have also concluded from a palaeontological evidence of extinctions in marine fauna at the K/T boundary that the mean temperature increased at, or immediately after, the extinction event. We note that the decrease in $^{13}\text{C}/^{12}\text{C}$ ratio of the planktonic foraminifera appears to extend for 10^4 – 10^5 yr after the K/T event³², in general agreement with the extinction timescale proposed by Hut *et al.*³³. This is considerably longer than the time interval of $< 10^3$ yr associated with deposition of the platinum-group-element layer. The extended 10^4 – 10^5 -yr extinction interval is not easily explained by theories of aerosol atmospheric loading or by a water-induced greenhouse effect. Our calculations indicate that an enhanced atmospheric greenhouse effect caused by a large impact-devolatilization-induced increase in atmospheric CO_2 would result in significant heating of the Earth, causing a lengthy period of mass extinctions. □

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1. Alvarez, L. E., Alvarez, W., Asaro, F. & Michel, H. V. *Science* **208**, 1095–1108 (1980).
2. Owen, M. R. & Anders, M. H. *Nature* **334**, 145–147 (1988).
3. Bohor, B. F., Foord, E. E., Modreski, P. J. & Triplehorn, D. M. *Science* **224**, 867–869 (1984).

4. Hess, J., Bender, M. L. & Schilling, J.-G. *Science* **231**, 979–984 (1986).
5. MacDougall, J. D. *Science* **239**, 485–487 (1988).
6. Bourgeois, J., Hansen, T. A., Wiberg, P. L. & Kauffman, E. G. *Science* **241**, 567–570 (1988).
7. Hildebrand, A. R. & Boynton, W. V. *Abstr. Conf. on Global Catastrophes in Earth History*, 78–79 (Lunar and planet. Inst., 1988).
8. Toon, O. B. et al. *Geol. Soc. Am. spec. Pap.* **190**, 187–200 (1982).
9. Gerstl, S. A. W. & Zardecki, A. *Geol. Soc. Am. spec. Pap.* **190**, 201–210 (1982).
10. Thompson, S. L., Ramaswamy, V. & Covey, C. J. *Geophys. Res.* **92**, 10942–10960 (1987).
11. Wolbach, W. S., Lewis, R. S. & Anders, E. *Science* **230**, 167–170 (1985).
12. Emiliani, C., Kraus, E. B. & Shoemaker, E. M. *Earth Planet. Sci. Lett.* **55**, 317–324 (1981).
13. Turco, R. P. et al. *Science* **214**, 19–23 (1981).
14. Lewis, J. S., Watkins, G. H., Hartman, H. & Prinn, R. *Geol. Soc. Am. spec. Pap.* **190**, 215–222 (1982).
15. O'Keefe, J. D. & Ahrens, T. J. *Geol. Soc. Am. spec. Pap.* **190**, 103–120 (1982).
16. Prinn, R. G. & Fegley, B. *Earth planet. Sci. Lett.* **83**, 1–15 (1987).
17. Lange, M. A. & Ahrens, T. J. *Earth planet. Sci. Lett.* **77**, 409–418 (1986).
18. O'Keefe, J. D. & Ahrens, T. J. *Proc. Lunar Sci. Conf.* **8th**, 3357–3375 (1977).
19. Poldervaart, A. *Geol. Soc. Am. spec. Pap.* **62**, 119–144 (1955).
20. Holland, H. D. *Chemistry of the Atmosphere and Oceans* (Wiley, New York, 1978).
21. Roddy, D. J. et al. *Int. J. Impact Engng* **5**, 525–541 (1987).
22. O'Keefe, J. D. & Ahrens, T. J. *Abstr. Lunar planet. Sci.* **XIX**, 887–888 (1988).
23. Walker, J. C. G., Hays, P. B. & Kastings, J. F. *J. geophys. Res.* **86**, 9776–9782 (1981).
24. Broecker, W. S. & Peng, T.-H. *Tracers in the Sea* 690 (Eldigio, New York, 1982).
25. Berner, R. A., Lasaga, A. C. & Garrels, R. M. *Am. J. Sci.* **283**, 641–683 (1983).
26. Hsü, K. J., McKenzie, J. A. & He, Q. X. *Geol. Soc. Am. spec. Pap.* **190**, 317–328 (1982).
27. Kastings, J. F. & Ackerman, J. P. *Science* **234**, 1383–1385 (1986).
28. Holser, W. T., Schildowski, M., Mackenzie, F. T. & Maynard, J. B. in *Chemical Cycles in the Evolution of the Earth* (eds Gregor, C. B., Garrels, R. M., Mackenzie, F. T. & Maynard, J. B.) 105–173 (Wiley, New York, 1988).
29. Zachos, J. C. & Arthur, M. A. *Paleoceanography* **1**, 5–26 (1988).
30. Gerstel, J., Thunell, R. C., Zachos, J. C. & Arthur, M. A. *Paleoceanography* **1**, 97–117 (1986).
31. McLean, D. M. *Science* **201**, 401–406 (1978).
32. Hsü, K. J. & McKenzie, J. A. *Geophys. Monogr.* **32** (Am. Geophys. Un., Washington DC, 1985).
33. Hut, P. et al. *Nature* **329**, 118–126 (1987).

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Parental care and mating behaviour of polyandrous dunnocks *Prunella modularis* related to paternity by DNA fingerprinting

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INDIVIDUALS are often assumed to behave so as to maximize their reproductive success¹ but unambiguous determination of parentage is difficult, especially in species with complex social systems where a female may mate with several males and where there may also be intraspecific brood parasitism^{2–4}. Even in apparently monogamous species, extra-pair paternity can be common^{5–7}. DNA fingerprinting^{8–11} promises to revolutionize field studies by providing a powerful method for determining paternity and maternity¹². Here we use this technique to link observations of mating behaviour and parental care with precise measurements of reproductive success. We show that in the dunnock *Prunella modularis*, a small passerine bird with a variable mating system^{13,14}, males do not discriminate between their own young and those of another male in multiply-sired broods. Nevertheless, they increase their own reproductive success by feeding offspring in relation to their access to the female during the mating period, which is a good predictor of paternity.

We studied a colour-ringed breeding population of about 80 dunnocks in the Cambridge University Botanic Garden^{14–16}. Some female territories were defended by one male (monogamy), others by two, unrelated males (polyandry). Sometimes two males jointly defended the territories of two or three adjacent females (polygynandry). Where two males shared the

defence of one or more female territories, one (alpha) was clearly dominant and able to displace the other (beta) from the female's vicinity¹⁴.

In 1988 we observed females from the time they completed nest building until the start of incubation (6–10 days), which is the period when copulations occur^{15,16} (mean observation time 4.02 h, $N = 34$). Monogamous males spent on average 81.1% of their time within 10 m of the female (range 62.1–97.3%, $N = 10$). Mate guarding was very effective; intruders were seen to gain exclusive access to the female (defined as the presence of only one male within 10 m) in only 3 of the 10 cases, a total of only 3 min in 23.98 h observation (mean for the 10 cases = 0.17% observation time).

By contrast, there was intense competition for matings when two males shared a female. Alpha males attempted to guard the female and prevent the beta male from gaining access, but this was difficult because beta males were often very persistent and, furthermore, females attempted to escape the alpha males' close attentions and encouraged beta males to mate¹⁶. In 8 out of 11 cases of polyandry and 10 out of 13 cases of polygynandry, the beta male had some exclusive access to the female. There was no significant variation in the proportion of exclusive access gained by alpha or beta males at different stages of the mating period and the mean copulation rate during periods of exclusive access was no different for alpha males (1.98 per h) and beta males (2.15 per h; $N = 16$; Wilcoxon matched pairs test, NS). Exclusive access time therefore gave a good measure of mating success and was easier to record than copulations. As in monogamy, intruding males gained very little exclusive access (only 3 out of 24 cases, a total of 5 min in 112.7 h; mean of 0.05% observation time).

As expected from our mating observations, DNA fingerprinting (Fig. 1) shows that intruding males gained little success; of 133 young (45 broods) all but one were fathered by a resident male. The exception is a case of polyandry where one chick from a brood of four was fathered by an alpha male from a neighbouring territory. All chicks from monogamous mating systems were sired by the resident male, whereas in polyandry and polygynandry paternity was often shared between alpha

TABLE 1 Analysis of paternity by DNA fingerprinting for three dunnock mating systems

Mating system	% Total young (n) fathered by		Mean % paternity per nest (n)		Details for each nest: proportion fathered by $\alpha\delta$
	$\alpha\delta$	$\beta\delta$	$\alpha\delta$	$\beta\delta$	
Monogamy (1 δ 1 φ)	100	— (49)	100	0 (15)	4/4, 4/4, 4/4, 4/4, 4/4, 4/4, 3/3, 3/3, 3/3, 3/3, 3/3, 3/3, 2/2, 2/2.
Polyandry (2 δ 1 φ)	52.9	44.1 (34)*	49.2	48.5 (11)*	4/4, 3/4*, 3/3, 2/2, 2/4, 1/4, 1/4, 1/3, 1/3, 0/2, 0/1.
Polygynandry (2 δ 2 φ ; 2 δ 3 φ)	72.0	28.0 (50)	68.4	31.6 (19)	3/3, 3/3, 3/3, 3/3, 3/3, 2/2, 2/2, 2/2, 1/1, 3/4, 3/4, 2/4, 2/4, 2/3, 1/3, 1/2, 0/2, 0/1, 0/1.

The proportions of young sired by the two males in polyandry and polygynandry are not significantly different. All but one of the 133 young were fathered by a male resident on the territory.

* One chick was fathered by a male on a neighbouring territory (see Fig. 1 legend).

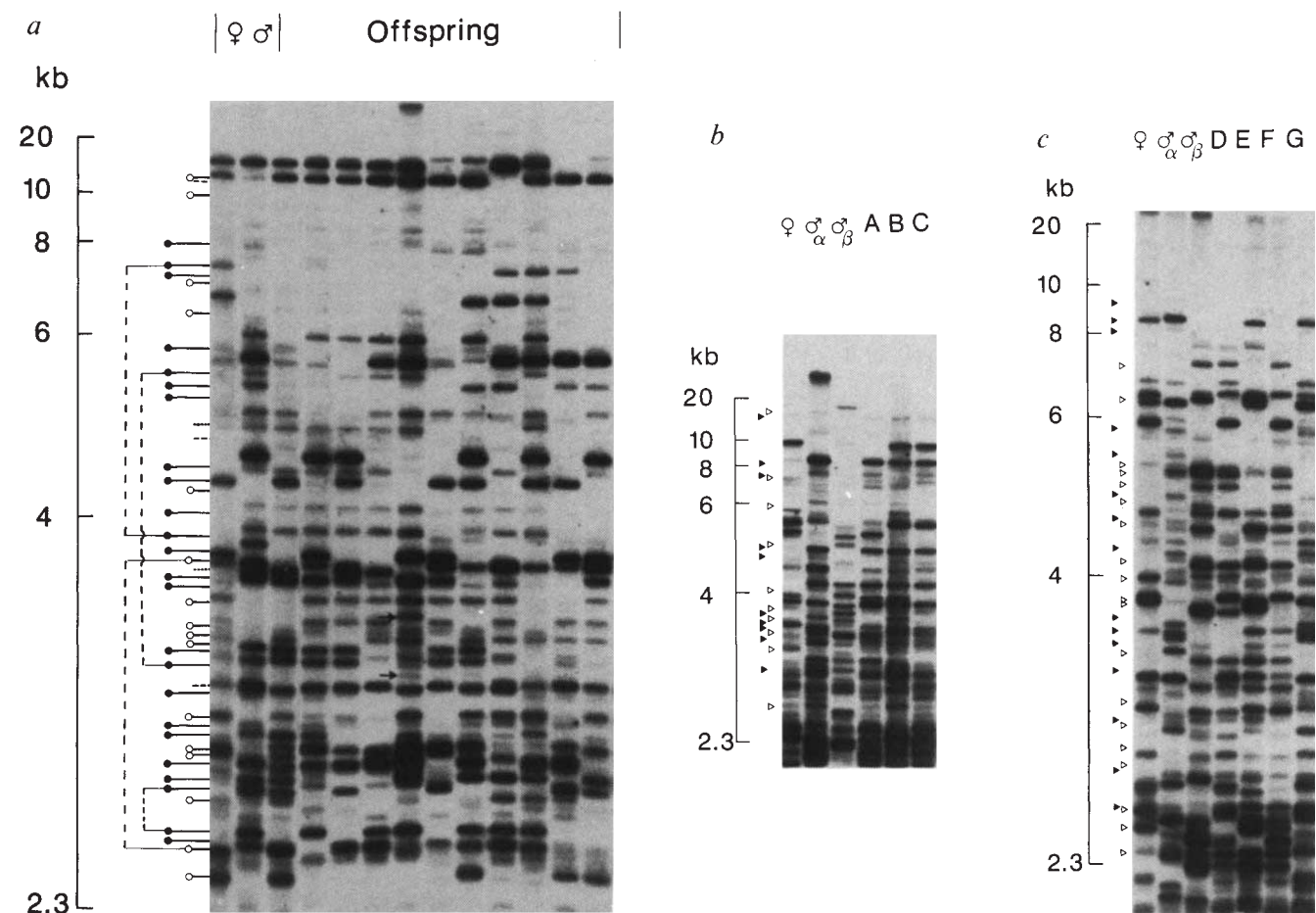


FIG. 1 Example dunlock DNA fingerprints obtained with probe 33.15 (ref. 8). *a*, Monogamous pair and 11 offspring (3 broods). DNA fragments of 'almost identical' (.....) and 'apparently identical' (----) mobility are indicated. Segregation analysis^{9,17} of the other 40 resolved maternal (○) and paternal (●) heterozygous fragments larger than 2.3 kb revealed a transmission frequency of 0.495, four apparently allelic pairs (joined by ---), and no consistent cosegregation; hence the bands probably represent dispersed hypervariable minisatellite DNA sequences^{9,17}. *b*, Polyandry (exclusive paternity): brood where alpha male sired all three nestlings A, B, C. *c*, Polyandry (mixed paternity): brood where beta male sired nestlings D, E, F, and alpha sired G. Markers indicate paternal male-specific (diagnostic) alpha (▶) and beta (▷) bands. METHODS. DNA was extracted from blood (40–100 μl, from brachial veins of adults and 7-day-old nestlings into 1 × SSC, 10 mM EDTA pH 7.0), digested with *AluI*, and fingerprints obtained as previously described⁹ except: 5 μg ethidium bromide was added per sample, gels were 0.8% (20-cm long) or 1.0% (30-cm long) agarose in 1 × TBE, and hybridizations, at 64 °C, excluded competitor DNA. The probability of band sharing (*x*) between individuals⁹ (conservatively found from 20-cm gels, including 'almost identical' bands) = 0.24 ± 0.10 s.d. (*N* = 17); bands per fingerprint (*n*) = 23.2 ± 4.1 s.d. (*N* = 29); hence mean allele frequency¹⁸ (*q*) = 0.13. For trios, fathers were initially assigned by

the presence of diagnostic bands in each nestling (range 2–13, mean 7.4 ± 2.5 s.d., *N* = 75). In three nestlings, one or two (arrowed in *a*) bands absent in both parents were considered mutant¹⁹ as band sharing was otherwise greater than expected for a misassigned random adult (*P*₁ < 0.01, *P*₂ < 0.001, *P*₃ < 0.05); apparent mutation rate = 10^{−4}. For the other 130 offspring *n* ≥ 12; it is extremely unlikely that all 12 fragments would be present in a random pair²⁰ as *P* = (1 − (1 − *x*)¹²)² = 3.3 × 10^{−5}. We concluded that no intraspecific brood parasitism, at least by non-relatives, occurred and that all mothers were correctly identified. Obligate paternal-derived bands could therefore be inferred in all offspring (range 5–18, mean 9.9 ± 2.8 s.d., *N* = 133). The probability of an unrelated male containing all these *m* fragments, *P* = *x*^{*m*}, in each case lay between 8.0 × 10^{−4} (*m* = 5) and 7.0 × 10^{−12} (*m* = 18); similarly for a close relative (brother, father and son)²¹, *P* = ((1 + *x*)/2)^{*m*} = 0.092 to 1.8 × 10^{−4}. Demographic data¹⁴ also shows that close relatives are very unlikely to become neighbours. In the one case of a nestling sired by an intruding male the female shared 14 of 25 offspring bands (*P* < 0.001) and the assigned male contained all 11 paternal specific bands (*P* < 2 × 10^{−7}), which is also significantly more than if he were the actual father's brother (expected band sharing⁹ = 1 − (1 − ((1 + *x*)/2(2 − *q*)))(1 − *q*) = 0.42, *P* < 0.001).

TABLE 2 Association between paternity, feeding of nestlings and access to the female during the mating period for alpha and beta males in polyandry and polygyny

Paternity (from DNA fingerprinting)	Behavioural observations			
	Male fed young	Male did not feed young	Male had some exclusive access to female	Male did not have exclusive access to female
Alpha male				
Broods where he had paternity	23	1	13	0
Broods where he had no paternity	2	3	4	1
	<i>P</i> = 0.011*		Sample size too small to test	
Beta male				
Broods where he had paternity	14	3	10	0
Broods where he had no paternity	4	8	2	6
	<i>P</i> = 0.011*		<i>P</i> = 0.005*	

Chick feeding by males was recorded for 29 out of the 30 nests in Table 1; we had observations during the mating period for 18 of these cases. We recorded 90% cases where beta males fed the young, and 85% cases where they had some exclusive mating access, within the first hour of observation, so observation times (see text) were sufficient to give reliable measures.

* Fisher exact probability test.

and beta male (Table 1). Maternity was assigned in all cases to the resident female, so there were no cases of intraspecific brood parasitism.

In all 15 cases of monogamy, the young were fed only by the monogamous male and female. In the 30 cases of polyandry and polygynandry where we recorded chick feeding, the female fed the young at all 30 nests, helped by both males in 14 cases, by the alpha male only in 11 cases (in one of these the beta male died during incubation), by the beta male only in 4 cases (in one of these the alpha male died) and in one case the female got no male help (mean observation time per nest = 4.35 h, $N = 45$).

DNA fingerprinting showed that males were more likely to help feed the young if they had paternity (Table 2). Two results indicate that this association does not arise because males can recognize their own offspring. First, in two cases the alpha male fed nestlings even though he had no paternity and in four cases the beta male did so. (In two cases both helped to feed a single chick.) Second, after fledging, broods were often divided among the parents with each adult taking sole care of some young for a further 2 weeks until they became independent. There was no tendency for males to pick out their own young. We observed 28 dependent fledglings, from 12 broods where both alpha and beta males had helped to feed the nestlings; 11 young were sired by the alpha male, of which 5 were fed by their father and 6 by the beta male, whereas 17 were sired by the beta male, of which 9 were fed by their father and 8 by the alpha male.

How, then, does the association between chick feeding and paternity come about? A male's access to the female gives a good prediction of his chances of paternity (Table 2), and males apparently use mating access to determine whether they feed young. Including data from all polyandrous and polygynandrous mating systems observed in 1981–88, beta males fed the young in 22 of the 27 cases where they had some exclusive access during the mating period, compared with only 1 out of 11 cases where they were not observed to have access ($P < 0.001$). Furthermore, beta males brought a greater proportion of the male feeds where they had had a greater proportion of the exclusive access time during the mating period (Fig. 2).

Females benefit by copulating with two males because chicks are fed more, and more survive with the help of two males rather than one²². An alpha male, however, would need at least 60–70% of the paternity in trio-fed broods for co-operative polyandry

to bring greater reproductive success than monogamy²³. For 20 broods where both males in polyandry and polygynandry had access to the female during the mating period, or both fed the young (and so, by implication, both had had access), the mean paternity split was 55.0% alpha, 45.0% beta (total of 58 young, 56.9% sired by the alpha male). This suggests that alpha males do not achieve the critical proportion above which it would pay them to share the female, and explains why although females encourage the presence of beta males, alpha males attempt to drive them away²³. □

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1. Clutton-Brock, T. H. (ed.) *Reproductive Success*. (Chicago University Press, 1988).
2. Brown, J. L. *Helping and Communal Breeding in Birds: Ecology and Evolution*. (Princeton University Press, 1987).
3. Wrege, P. H. & Emlen, S. T. *Behav. Ecol. Sociobiol.* **20**, 153–160 (1987).
4. Koenig, W. D. & Mumme, R. L. *Population Ecology of the Cooperatively Breeding Acorn Woodpecker*. (Princeton University Press, 1987).
5. Sherman, P. W. & Morton, M. L. *Behav. Ecol. Sociobiol.* **22**, 413–420 (1988).
6. Westneat, D. F. *Anim. Behav.* **35**, 877–886 (1987).
7. Gowaty, P. A. & Karlin, A. A. *Behav. Ecol. Sociobiol.* **15**, 91–95 (1984).
8. Jeffreys, A. J., Wilson, V. & Thein, S. L. *Nature* **314**, 67–73 (1985).
9. Burke, T. & Bruford, M. W. *Nature* **327**, 149–152 (1987).
10. Wetton, J. H., Carter, R. E., Parkin, D. T. & Walters, D. *Nature* **327**, 147–149 (1987).
11. Burke, T. *Trends Ecol. Evol.* (in the press).
12. Brookfield, J. F. Y. *I.M.A. J. math. appl. Med. Biol.* (in the press).
13. Snow, B. & Snow, D. J. *Yamashina Inst. Ornith.* **14**, 281–292 (1982).
14. Davies, N. B. & Lundberg, A. J. *anim. Ecol.* **53**, 895–912 (1984).
15. Davies, N. B. *Nature* **302**, 334–336 (1983).
16. Davies, N. B. *Anim. Behav.* **33**, 628–648 (1985).
17. Jeffreys, A. J., Wilson, V., Thein, S. L., Weatherall, D. J. & Ponder, B. A. J. *Am. J. hum. Genet.* **39**, 11–24 (1986).
18. Jeffreys, A. J., Wilson, V. & Thein, S. L. *Nature* **316**, 76–79 (1985).
19. Jeffreys, A. J., Royle, N. J., Wilson, V. & Wong, Z. *Nature* **332**, 278–281 (1988).
20. Jeffreys, A. J., Brookfield, J. F. Y. & Semeonoff, R. *Nature* **317**, 818–819 (1985).
21. Hill, W. G. *Nature* **322**, 290–291 (1986).
22. Davies, N. B. *J. anim. Ecol.* **55**, 123–138 (1986).
23. Davies, N. B. & Houston, A. I. *J. anim. Ecol.* **55**, 139–154 (1986).

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Incubation period of AIDS in San Francisco

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IN a closed population, the distribution of AIDS diagnoses over time is the convolution of the distributions of human immunodeficiency virus (HIV) infections and the incubation period. This has motivated estimates of the infection distribution, assuming known diagnosis and incubation distributions¹, but the usefulness of this method is limited by uncertainty about incubation^{2–4}. The large amount of information on the distribution of HIV infections in San Francisco's gay community suggests the opposite approach—estimating the incubation distribution, assuming known infection and diagnosis distributions. A non-parametric implementation of this strategy produced an estimate with a median at 9.8 years, increasing hazard rates, and less uncertainty than previous estimates.

Data are available for seroconversions rather than infections, so we estimated the time from seroconversion to AIDS diagnosis⁵. (Seroconversion may occur later than infection⁶.) Figure 1a shows estimated seroconversion rates from three sources, and Fig. 1b shows the overall seroconversion and diagnosis patterns that we used. The dramatic drop in seroconversion by 1984 was paralleled by rectal gonorrhea rates among males at the same time⁷, presumably because of behaviour change. The effect of such change on HIV infections would have been enhanced by the saturation of high-risk subgroups⁸. This further

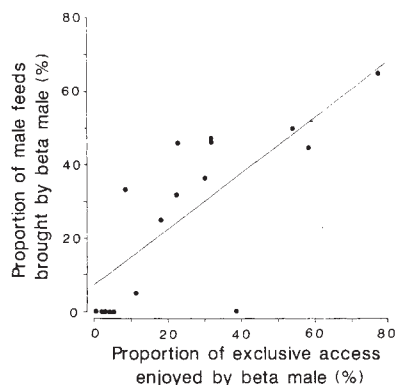


FIG. 2 Relationship, for polyandry and polygynandry, between the proportion of exclusive access time with the female by the beta male during the mating period, and the proportion of male feeds brought by the beta male to the nestlings. Exclusive access time is the total time that either the alpha or beta male was the only male within 10 m of the female. Data from 1981–88 for cases where beta males gained some exclusive access ($y = 5.75 + 0.798x$; analysis on log-transformed data: $r = 0.775$, 15df, $P < 0.01$; the slope does not differ significantly from 1, $t = 1.33$, 15df). In polygynandry, a male's provisioning of the young at one nest may be influenced by the activities of other females in the mating system^{16,22} so cases where other females were laying or had young simultaneously were excluded.

TABLE 1 Estimates of the incubation period of AIDS

Estimate	Median (yr) (95% CI)	Per cent with AIDS by 9 yr (95% CI)
Present study	9.8 (9.4, ∞)	45 (42, 47)
Penalized 15% for uncertainty in seroconversion pattern	(8.6, ∞)	(36, 54)
Parametric analysis of US transfusion data ³	7.3 (4.6, ∞)	Not given
Parametric analysis of data from 84 men in the San Francisco City Clinic Cohort ²	7.8 (4.2, 15.0)*	65 (not given)
Nonparametric analysis of data from 181 men in the San Francisco City Clinic Cohort ¹⁵	Not given	42 (30, 54) [†]

* Estimated mean and 90% confidence interval (CI).

[†] Confidence interval does not reflect uncertainty resulting from imputing exact seroconversion times from interval-censored data.

corroborates the drop shown by all three sources in Fig. 1a. Mathematical models of epidemic spread in a susceptible population⁸ corroborate the early rapid rise.

Treating the distributions in Fig. 1b as known allows estimation of the incubation distribution. In particular, we wish to estimate parameters h_k , the hazard rates for developing AIDS k months after seroconversion. With 10 years worth of data, h_k can be estimated for $k=0$ to 120. Let $d_{j,k}$ be the number of persons who seroconverted in month j and were diagnosed in month k . If all the $d_{j,k}$ were known, then the maximum likelihood estimate of h_k would be

$$\hat{h}_k = e_k / r_k \quad (1)$$

where $e_k = \sum_{j=0}^{120-k} d_{j,j+k}$ is the number of persons diagnosed exactly k months after seroconversion, and r_k is the number of

persons still free of AIDS ($k-1$) months after seroconversion. Although the $d_{j,k}$ are unknown, we can use the EM algorithm⁹ to estimate the h_k . The algorithm begins with arbitrary initial values of the h_k and calculates the expected values of the $d_{j,k}$ conditional on the initial h_k and the known seroconversion and diagnosis patterns. The expected values are used in place of the unknown $d_{j,k}$ in equation (1) to yield refined estimates of the h_k . The refined h_k s lead to new expected values, which yield new estimated h_k s, and so on. The algorithm continues until the estimates converge.

Because it is biologically reasonable that the hazard changes smoothly over time, we used a modification of equation (1):

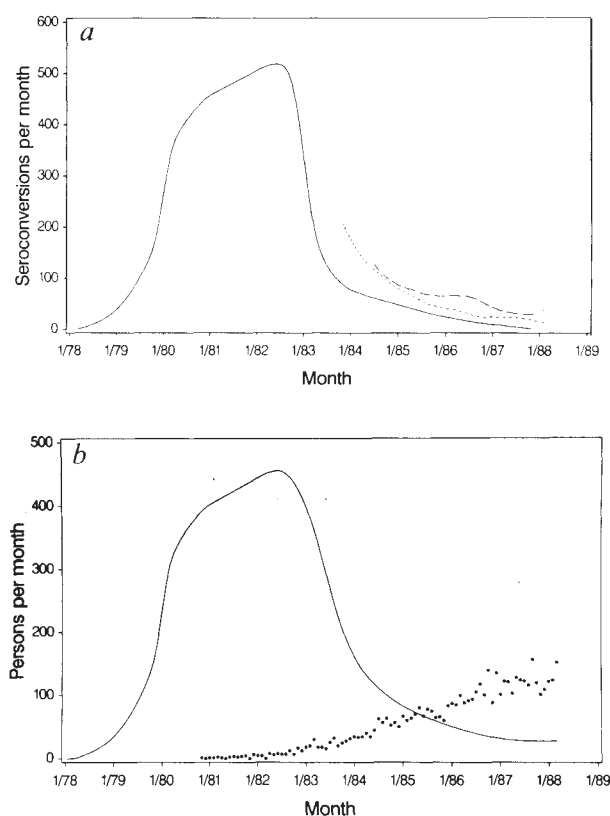
$$\hat{h}_k = \frac{\sum_{i=k-6}^{k+6} e_i}{\sum_{i=k-6}^{k+6} r_i} \quad (2)$$

This is a local likelihood estimate¹⁰ with a span of 13. (This span was the smallest that produced acceptably smooth estimates.) In addition to ensuring reasonable smoothness, this modification also reduced the estimate's variability and led to faster convergence, while still being non-parametric. Figure 2 shows the estimates that resulted from applying this method to the distributions in Fig. 1b. The estimated probabilities of developing AIDS during the first to tenth years following seroconversion were 0.002, 0.007, 0.022, 0.043, 0.061, 0.073, 0.082, 0.081, 0.074 and 0.067, respectively. The estimated distribution is an average for all gay men in San Francisco. It may well differ for subgroups of this population, and it may change as prophylaxis of seropositives becomes more widespread before diagnosis.

The confidence intervals in Fig. 2 do not reflect the uncertainty that results from estimation of the seroconversion pattern. Multiplying the seroconversion curve in Fig. 1b by a factor (or dividing the diagnosis curve by a factor) would divide the curve in Fig. 2a by the same factor. As the estimated number of seroconversions may be too high or too low by 10% (ref. 11), the curve in Fig. 2a could plausibly be multiplied by 0.9 or 1.1.

FIG. 1 Data used for estimating the distribution of times from HIV seroconversion to AIDS among gay men in San Francisco. a, HIV seroconversions estimated from the hepatitis B vaccine trial (solid line), the San Francisco Men's Health Study¹¹ (long dashes), and the San Francisco General Hospital Study¹² (short dashes). b, Overall estimates of HIV seroconversions (solid line) and AIDS diagnoses (dots).

METHODS. a, For the hepatitis B vaccine trial, a smooth curve was fitted to match estimated yearly totals¹⁶ and scaled to total 20,260 seroconversions through September 1984. The total 20,260 is an estimated 8,757 seropositive but AIDS-free men from a random sample of part of San Francisco¹¹, divided by the proportion of AIDS cases from October 1984 until July 1988 who came from the sampled area (1,708 of 3,767, or 45%; this proportion was fairly stable over the period) plus 800 AIDS cases diagnosed up to September 1984. For the other cohorts, Turnbull's EM algorithm¹³ was applied to interval-censored seroconversion data, with local-likelihood smoothing¹⁰ in the M-step. The curves were scaled by the same factor as the vaccine trial estimate. b, Monthly diagnosis totals up to March 1988 are from surveillance data as of July 1988, adjusted for several sources of error. Based on data reported of November 1987 until July 1988, the proportionate increase in monthly totals arising from reports more than four months after diagnosis was modelled as a quadratic function of difference between report and diagnosis month, plus a quadratic function of calendar month of report (to allow for diminishing influence of the change in case definition⁵). This yielded corrections ranging from +14% for diagnoses in March 1988 to less than +1% for March 1987 and before. The San Francisco Department of Public Health has estimated under-reporting as ~2% before 1986 and ~5% more recently, and has also estimated that about 4% of San Francisco residents with AIDS are diagnosed elsewhere (unpublished data). Follow-up of the San Francisco General Hospital Cohort¹², in which 3 of 69 AIDS cases (4%) were diagnosed after moving from the city, suggested a correction of +4% for persons included in the seroconversion totals who were not residents when diagnosed. The seroconversion curve is a compromise among the curves from (a) that retains the overall shape from the hepatitis B vaccine trial and agrees closely with the other curves, while remaining smooth and totalling 20,260 seroconversions up to September 1984.



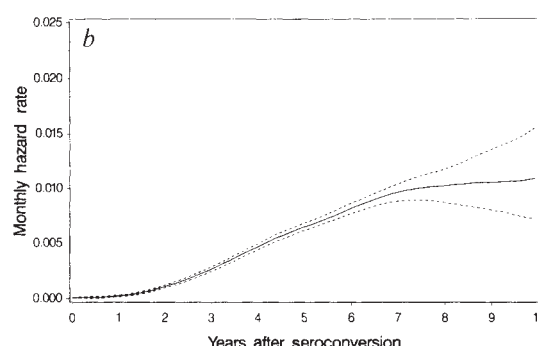
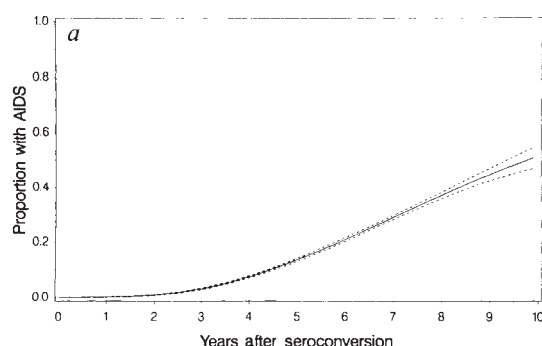


FIG. 2 Estimated distribution of time from HIV seroconversion to diagnosis of AIDS for gay men in San Francisco, 1978–1988. *a*, Cumulative distribution function (solid line), with approximate 95% confidence intervals (dashed lines). *b*, Hazard function (solid line), with approximate 95% confidence intervals (dashed lines).

METHODS. Confidence intervals were determined by a bootstrap method¹⁴.

Shifting the seroconversion curve relative to the diagnosis curve would expand or contract the initial period of virtually no progression to AIDS. Applying the estimation method to a seroconversion pattern identical to the one in Fig. 1*b*, but with 10 seroconversions per month from December 1976 to May 1978, would change the estimate in Fig. 2*a* by less than 0.006 at all time points, so the possibility of an unobserved long, slow start to the epidemic does not invalidate the estimate.

Figure 3 compares fitted monthly AIDS diagnosis totals with adjusted surveillance data. The fitted curve matches the data well. The projected curves reflect the range of distributions shown in Fig. 2*b*. They are not influenced by uncertainty in the magnitude of the seroconversion curve, because any error would result in an opposite error in the incubation estimate, with no net influence on projected totals. The projections show the sharp drop in seroconversions in Fig. 1*b* manifesting itself as a much more gradual drop in AIDS diagnoses 6 to 8 years after the drop in seroconversions. For each of the three projections, the time between the peaks in seroconversions and projected diag-

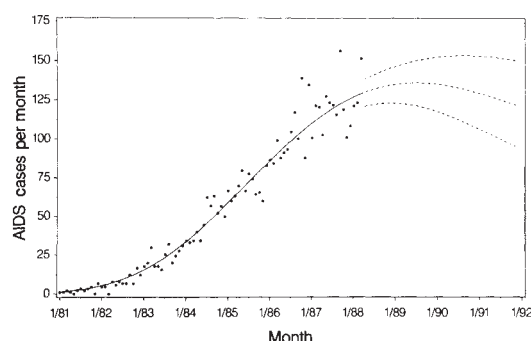


FIG. 3 Fitted (solid line) and projected (dashed lines) AIDS diagnoses among gay men in San Francisco. Projections are based on incubation distributions corresponding to the estimate in Fig. 2*b*, with the hazard remaining constant beyond year 10 (middle projection), and corresponding to the upper and lower confidence limits in Fig. 2*b*, with the hazard extrapolated linearly beyond year 10 (upper and lower projections). The adjusted surveillance totals from Fig. 1*b* (dots) are shown for comparison. Note that the vertical scale represents corrected rather than raw surveillance totals.

METHODS. Given v_j seroconversions in month j and probability p_k of developing AIDS k months after seroconversion, the expected number of diagnoses in month i is $\sum_{j=0}^i v_j p_{i-j}$. Fitted and projected monthly diagnosis totals were calculated by this formula with the v_j s from the seroconversion curve in Fig. 1*b* (with constant extrapolation through 1991) and the p_k s from the incubation distributions described above.

The estimated incubation distribution was used to generate simulated diagnosis data assuming the seroconversion pattern shown in Fig. 1*b*. Estimates were made on 200 such sets of simulated data to assess the variability resulting from random variation in how well the diagnosis pattern reflects the incubation distribution. At each month, 95% of the estimates fell between the dashed lines.

noses is approximately equal to the time of the peak in the density of the incubation. The gradualness of the drop in projected diagnoses is due to the large variability in incubation times⁸.

Four factors may bias the estimate of Fig. 2 upwards when compared with other studies: use of the more inclusive 1987 definition of AIDS⁵, use of seroconversion rather than infection times, inclusion of cases who seroconverted outside San Francisco in diagnosis totals but not in seroconversion totals, and possible downward bias in the magnitude of the seroconversion curve resulting from over representation of seropositives from the sampled area¹¹ among diagnosed AIDS cases. Despite these factors, Fig. 2 shows slower progression to AIDS than previous estimates^{2,3} (Table 1). The large uncertainty in previous estimates, however, makes them quite compatible with Fig. 2. In addition, analyses based on blood-transfusion data deal with a very different population and only estimate the incubation period that is conditional on AIDS developing.

The incubation period of AIDS is difficult to study. Prospectively followed incident seroconverters are rare, so estimates based on them are highly uncertain², and data from transfusion-associated AIDS cases are inherently limited³. The method used here is an alternative approach. It is possible because of the availability of information on seroconversions among gay men in San Francisco and because the drop in seroconversions makes the pattern of diagnoses quite informative about the incubation. Its dependence on an accurate estimate of seroconversions is a drawback, but it does not rely on parametric assumptions, and Table 1 shows that the estimate is more precise than others, even allowing for uncertainty in estimating seroconversions.

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1. Brookmeyer, R. & Gail, M. H. *J. Am. statist. Ass.* **83**, 301–308 (1988).
2. Lui, K.-J., Darrow, W. W. & Rutherford, G. W. *Science* **240**, 1333–1335 (1988).
3. Kalbfleisch, J. D. & Lawless, J. F. *Nature* **333**, 504–505 (1988).
4. Medley, G. F., Anderson, R. M., Cox, D. R. & Billard, L. *Nature* **333**, 505 (1988).
5. Centers for Disease Control *Morbidity Mortal Wkly Rep.* **36**, 1S–15S (1987).
6. Rankin, A. *et al. Lancet* **ii**, 589–593 (1987).
7. Pickering, J. *et al. Math. Modelling* **7**, 661–668 (1986).
8. Anderson, R. M., May, R. M., Medley, G. F. & Johnson, A. *IMA J. math. med. Biol.* **3**, 229–263 (1986).
9. Dempster, A. P., Laird, N. M. & Rubin, D. B. *J. R. statist. Soc. Ser. B* **39**, 1–22 (1977).
10. Tibshirani, R. & Hastie, T. *J. Am. statist. Ass.* **82**, 559–567 (1987).
11. Winkelstein, W. *et al. J. Am. med. Ass.* **257**, 321–325 (1987).
12. Moss, A. R. *et al. Br. Med. J.* **296**, 745–750 (1988).
13. Turnbull, B. W. *J. R. statist. Soc. Ser. B* **38**, 290–295 (1976).
14. Efron, B. *Biometrika* **72**, 45–58 (1985).
15. Hessel, N., *et al. IV Int. Conf. AIDS*, 1988. Abstr. 4096.
16. Hessel, N., *et al. IV Int. Conf. AIDS*, 1988. Abstr. 4614.

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The HIV-1 *rev* trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA

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HUMAN immunodeficiency virus type 1 (HIV-1) replication requires the expression of two classes of viral mRNA. The early class of HIV-1 transcripts is fully spliced and encodes viral regulatory gene products. The functional expression of one of these nuclear regulatory proteins, termed Rev (formerly Art or Trs), induces the cytoplasmic expression of the incompletely spliced, late class of HIV-1 mRNAs that encode the viral structural proteins, including Gag and Env¹⁻⁶. Here, we provide evidence that this induction reflects the export from the cell nucleus to the cytoplasm of a pool of unspliced viral RNA constitutively expressed in the nucleus. The hypothesis that Rev acts on RNA transport, rather than splicing, is further supported by the observation that the cytoplasmic expression of a non-spliceable HIV-1 *env* gene sequence is also subject to Rev regulation. Here we show that this Rev response requires a specific target sequence which coincides with a complex RNA secondary structure present in the *env* gene. The response to Rev is fully maintained when this sequence is relocated to other exonic or intronic locations within *env* but is ablated by inversion. These results indicate that the HIV-1 *rev* gene product induces HIV-1 structural gene expression by activating the sequence-specific nuclear export of incompletely spliced HIV-1 RNA species.

In the absence of Rev, the genomic HIV-1 *tat* gene expression vector pgTAT⁶ (Fig. 1a) predominantly expresses a fully spliced cytoplasmic *tat* mRNA (Fig. 1b, lane 9) that encodes the 86-residue form of the HIV-1 Tat protein (Fig. 1c, lane 1). In the presence of Rev, pgTAT produces a high level of an unspliced cytoplasmic mRNA (Fig. 1b, lane 10), structurally equivalent to *env* mRNA⁶, that encodes a truncated, 72-residue Tat protein (Fig. 1c, lane 2). Because it has been proposed that Rev suppresses the splicing of HIV-1 transcripts^{1,7}, we examined the effect of substituting heterologous splice donor (pInsSD) or splice acceptor and branch-point sequences (pInsSA) for the authentic pgTAT splice sites (Fig. 1a). In each case, these sequences were derived from the genomic rat preproinsulin II gene⁸, which is not itself responsive to Rev (see below). Nuclease S₁ protection analysis using probes which spanned the insulin gene splice donor (Fig. 1b, lanes 1–4) or splice acceptor (Fig. 1b, lanes 5–8) reveals that the transfected pInsSA and pInsSD vectors predominantly express spliced cytoplasmic mRNA in the absence of Rev. Although these chimaeric transcripts used the predicted insulin RNA splice sites (Fig. 1b, lanes 1, 3, 6, 7) co-expression of *rev* nevertheless induces a high level of unspliced cytoplasmic mRNA (Fig. 1b, lanes 4, 8). In the case of pInsSA, the induced expression of an unspliced cytoplasmic *tat* mRNA was confirmed by immunoprecipitation analysis (Fig. 1c, lanes 3, 4).

The retention of full Rev responsiveness by pInsSA and pInsSD showed that the *cis*-acting Rev response element (RRE) must lie within the intronic *env* sequences of pgTAT. A series of deletion (pΔ) mutants of pgTAT (Fig. 1a) revealed that

vectors retaining an intronic BglIII (B2) to HindIII (Hd) fragment also retained full Rev responsiveness (Table 1). All vectors lacking this sequence, however, exclusively express the 86-residue Tat encoded by the spliced *tat* mRNA, regardless of *rev* co-expression. To localize more precisely the RRE within the B2/Hd fragment, we reinserted a series of subfragments into pΔB2/Hd to generate the pi vectors (Fig. 1a). The responsiveness of these vectors to Rev (Table 1) showed that the RRE must be at least 104 and possibly more than 210 nucleotides (nt) in length (Fig. 1a). Reinsertion of the complete B2/Hd fragment in the inverted orientation (piH/A) fails to restore Rev responsiveness (Fig. 1b, lane 16).

To determine whether the function of the RRE depends on location, we reinserted the B2/Hd fragment at selected sites within the Rev non-responsive pΔB2/Hd vector (Fig. 1a). These relocation (pλ) vectors reveal that the RRE is fully functional both at other intronic sites (pλK and pλB1, Table 1) and when inserted at an exonic BamHI (Bm) site located 3' to the *tat* gene splice acceptor (pλBm) (Fig. 1c, lanes 9, 10). Insertion at an EcoRI (R) site located immediately 3' to the vector polyadenylation site (pλR) however, did not restore Rev responsiveness to pΔB2/Hd (Fig. 1c, lanes 11, 12). It seems therefore that the RRE can function at any location within the primary *tat* gene transcript when present in the sense orientation.

The minimal size of the RRE is relatively large, indicating that secondary structure, in addition to primary sequence, may be important for recognition of the RRE. Indeed, the sequence encoding the N terminus of the HIV-1 gp41 Env protein is consistent with a high level of secondary structure⁹, and in Fig. 2 we present a stable predicted RNA stem-loop structure, which fully encompasses the minimal RRE defined by mutational analysis. The extension of this 234-nt secondary structure beyond the minimal 210-nt RRE defined by piD/G and piB/G may explain the incomplete Rev responsiveness of these vectors (Table 1). Interestingly, the only other predicted structure of comparable significance within the HIV-1 genome coincides with the putative RNA target sequence for *trans*-activation by the viral *tat* gene product⁹⁻¹¹.

Although the data indicate that the *rev* gene product regulates the cytoplasmic expression of unspliced HIV-1 mRNA by directly or indirectly interacting with an RNA secondary structure, the observation that the RRE can function at both exonic and intronic locations argues against a direct effect on viral

TABLE 1 Mutational definition of the HIV-1 Rev response element

Clone tested	Rev response	Clone tested	Rev response
pgTAT	+++	Reinsertions	
Substitutions		piA/H	+++
pInsSD	+++	piB/H	+++
pInsSA	+++	piB/G	++
Deletions		piB/E	—
pΔK/B1	+++	piC/F	—
pΔK/B2	+++	piD/G	++
pΔK/Hd	—	piA/D	—
pΔB1/B2	+++	piE/H	—
pΔB1/Hd	—	Inversions:	
pΔB2/Hd	—	piH/A	—
Relocations:			
pλK	+++		
pλB1	+++		
pλBm	+++		
pλR	—		

Different mutants of the parental pgTAT vector (Fig. 1a) were assayed for the Rev-induced expression of unspliced cytoplasmic *tat* mRNA by S₁ used elsewhere analysis (Fig. 1b) and immunoprecipitation (Fig. 1c). +++, full Rev response; ++, partial Rev response; —, no detectable Rev response. All constructions displayed the same phenotype as pgTAT in the absence of Rev coexpression.

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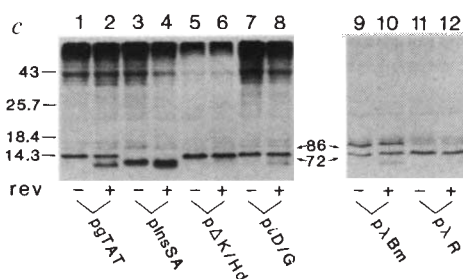
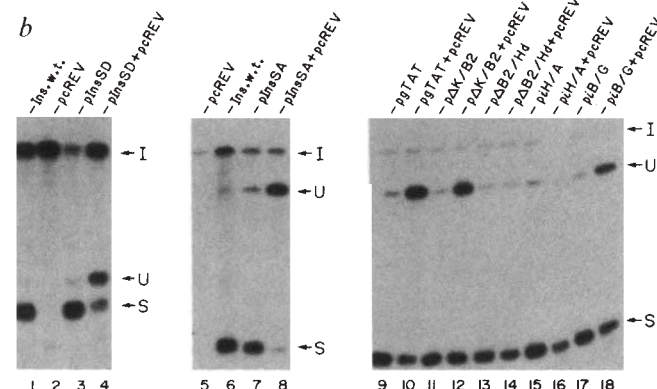
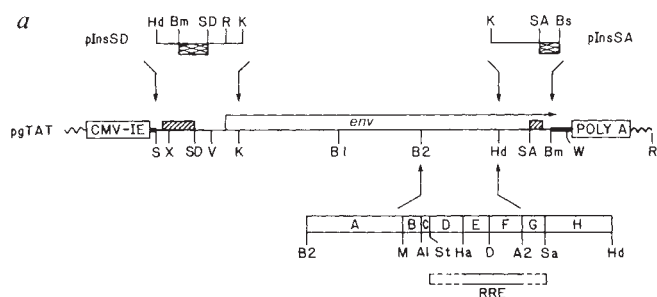


FIG. 1 Mapping of the HIV-1 Rev response element (RRE). **a**, The Rev responsive *tat* expression vector pgTAT contains the two HIV-1 *tat* gene-coding exons (hatched) separated by an *env*-gene-derived intronic element⁶. pInsSD and pInsSA were constructed by replacement of the authentic HIV-1 splice donor and splice acceptor elements with equivalent sequences from the genomic rat preproinsulin II gene expression vector pBC12MI⁸. Deletion (pΔ) mutants were constructed by excision of sequences between intronic restriction-enzyme sites. The pΔB2/Hd deletion mutant served as substrate for construction of the reinsertion (pi) and relocation (pλ) mutants. The names of the pi derivatives indicate which inclusive subfragment was reinserted. The pλ mutants were constructed by insertion of the complete A/H fragment at novel sites indicated by the vector name. The pENV160 vector lacks the HIV-1 SD sequence and instead proceeds from an *Avall* (V) site located immediately 5' to the *env* gene ATG³. pENV160 also contains 3' *env* gene sequences which overlap with, and extend 3' to, *rev* gene-coding sequences deleted from pgTAT. HIV-1 sequence coordinates (strain HXB-3) are relative to the start of the HIV-1 sequence at *Sall* at position 5,367 (ref. 18). S, *Sall*, 2; X, *Xho*I, 58; SD, splice donor, 260; V, *Avall*, 387; *env* initiation codon, 482; K, *Kpn*I, 564; B1, *Bgl*II, 1,254; B2, *Bgl*II, 1,834; M, *Mbo*I, 1,957; A1, *Alu*I, 1,986; St, *Sty*I, 1,994; Ha, *Hae*III, 2,058; D, *Dde*I, 2,103; A2, *Alu*I, 2,162; Sa, *Sau*3A1, 2,201; Hd, *Hind*III, 2,354; SA, Splice acceptor, 2,590; Bm, *Bam*HI, 2,688. Bs, *Bst*EII; W, *A*1w1; R, *Eco*RI. **b**, Quantitative S₁ nuclease protection analysis of cytoplasmic RNA isolated from COS cell cultures transfected with the indicated expression vectors^{6,8}. Transfections were performed in the presence of the *rev* expression vector pcREV or the negative control vector pBC12/CMV^{6,8}. The end-labelled, input probes (I) were designed to permit visualization of both unspliced (U) and spliced (S) cytoplasmic mRNA species. Ins. w.t., cultures transfected with the wild-type preproinsulin II gene expression vector pBC12MI (ref. 8). **c**, Immunoprecipitation analyses of transfected COS cell cultures were performed using anti-*tat* antisera⁶. Vectors were transfected in the presence (+) or absence (−) of the *rev* expression pcREV (ref. 6). The spliced pgTAT mRNA encodes an 86-residue protein whereas the unspliced mRNA encodes a 72-residue protein⁶. pInsSA lacks the second *tat* exon (Fig. 1a) and encodes a spliced mRNA predicted to give an 84-residue chimaeric Tat protein.

METHODS. **b**, The strategy for the S₁ nuclease protection analyses has been described⁶. In each case, the DNA probe was end-labelled within a coding exon and extended into the flanking intron. A pBR322-derived tag permits the distinction of the input probe from the unspliced RNA signal. The probe used in Fig. 1b, lanes 1–4 was labelled at an insulin gene *Bam*HI (Bm) site using Klenow DNA polymerase I (Fig. 1a) and extends through the insulin gene splice donor (SD) at 174 nt (S) to an intronic *Eco*RI (R) site at 193 nt (U). The input probe was 386 nt in length. The probe used in Fig. 1b, lanes 5–8 was end-labelled at an insulin gene *A*1w1 (W) site using polynucleotide kinase and extends through the insulin splice acceptor (SA) at 110 nt (S), to an intronic *Kpn*I (K) site at 378 nt (U). The input probe was 784 nt in length. The probe used in Fig. 1b, lanes 9–18 was end-labelled at a *tat* gene *Xho*I (X) site using Klenow DNA polymerase and extends through the *tat* gene splice donor (SD) at 202 nt (S) to an intronic *Kpn*I (K) site at 506 nt (U). The input probe was 798 nt in length.

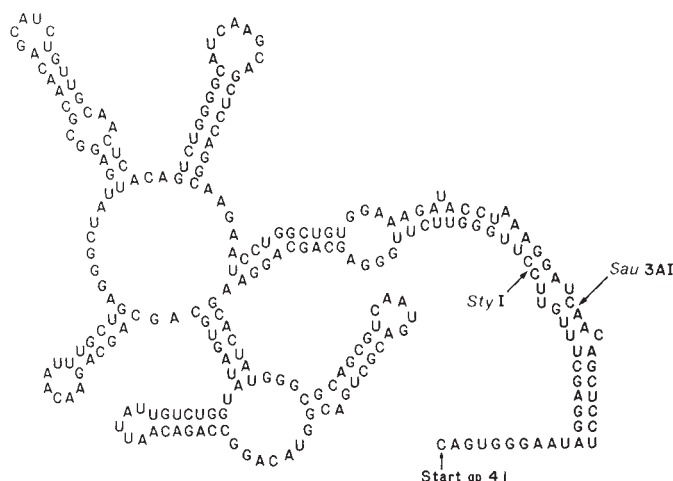


FIG. 2 Predicted secondary structure of the HIV-1 Rev response element. The mutationally defined minimal RRE extends from a 5' *Sty*I site to a 3' *Sau*3A1 site (pΔD/G, Fig. 1a). This sequence is located immediately 3' to the start of the gp41 *env* gene-coding region and coincides with the most highly conserved sequence domain within the *env* gene⁹. The RNA stem-loop structure presented is both extremely stable ($\Delta G = -115.1$ kcal/mol⁻¹) and highly significant (segment score in standard deviation units = -5.29). In a previous survey, no equivalent predicted secondary structure was observed within the HIV-1 genome⁹ and the RRE is therefore likely to be unique. In fact, the RRE would be contained within all known incompletely spliced HIV-1 mRNAs, including the *gag-pol* and *env* mRNAs.

METHODS. The distribution of significant secondary structures in the HIV-1 *env* gene was calculated for window (segment) sizes ranging from 30 to 300 bases by adding two bases to the segment size from 30 to 100 bases and five bases to the segment size from 100 to 300 bases. For each segment size, the calculation was carried out by sliding one base along the *env* gene-coding sequence. The segment scores which measure the statistical significance of the optimal secondary structures were computed⁹ using the Cray operating system of a CRAY X-MP/24 computer and the energy rules of Salser¹⁹ and Cech *et al.*²⁰.

RNA splicing. To examine the alternative hypothesis, that Rev activates the nuclear export of unspliced HIV-1 RNAs⁶, we used S_1 nuclease analysis to quantify the effect of Rev on the level of spliced and unspliced *tat* RNA within the total, nuclear and cytoplasmic fractions of cells transfected with pgTAT. As an internal control, these cultures were cotransfected with a genomic rat preproinsulin II gene expression vector⁸. This experiment again showed that unspliced *tat* mRNA contributes only a low percentage of the cytoplasmic steady-state mRNA in the absence of Rev (Fig. 3a, lane 6) whereas unspliced cytoplasmic *tat* mRNA predominates in *rev*-expressing cells (Fig. 3a, lane 7). In contrast, unspliced *tat* RNA was the predominant *tat* RNA species detected in the nucleus regardless of *rev* co-expression (Fig. 3a, lanes 4, 5), indicating that Rev does not significantly affect either the nuclear stability or splicing of HIV-1 transcripts. Interestingly, the level of cytoplasmic unspliced *tat* mRNA expression induced by Rev closely mirrored the high level of unspliced RNA constitutively expressed in the nucleus. This is consistent with the hypothesis that Rev functions to equilibrate the cytoplasm with a pre-existing pool of nuclear viral RNA. The Rev protein also has little effect on the level of unspliced mRNA in total cellular RNA (Fig. 3a, lanes 2, 3), suggesting that the nuclear pool of HIV-1 RNA in these transfected cells contributes a surprisingly high percentage of total cellular HIV-1 RNA. A parallel analysis of insulin RNA expression (Fig. 3b) reveals that Rev had no detectable effect on the relative expression of spliced and unspliced insulin transcripts. By contrast with the simultaneously expressed *tat* RNA, unspliced insulin RNA was observed at only a very low steady-state level and was excluded from the cytoplasm. The high steady-state level of unspliced nuclear *tat* RNA in these transfected cells indicates that this HIV-1 RNA is poorly recognized by the nuclear splicing machinery. For other retroviruses, sequences have in fact been defined which act constitutively and in *cis* to reduce splicing efficiency^{12,13}.

If the Rev protein acts to regulate the export from the nucleus to the cytoplasm of unspliced HIV-1 transcripts then it might also regulate the expression of genomic HIV-1 sequences which are incapable of splicing. In fact, Knight *et al.*³ have previously shown that an *env* gene expression vector (pENV160, see Fig. 1a legend) which encodes a non-spliceable *env* RNA, remains fully subject to regulation by Rev³. Furthermore, the Rev induc-

tion of *env* protein is not accompanied by a significant increase in total cellular *env* RNA³. Our results (Fig. 3a) however, suggest, that the latter observation could have resulted from the use of unfractionated RNA in the earlier study. To clarify this, we transfected COS cells with pgTAT or with pENV160 in the presence or absence of Rev. Cytoplasmic RNA from these cultures was then subjected to northern analysis using an *env*-gene-specific probe. As previously reported⁶, unspliced cytoplasmic pgTAT RNA was observed only in the presence of Rev (Fig. 3c, lanes 3, 4). Remarkably, the cytoplasmic expression of the *env* mRNA encoded by pENV160 was also observed to be fully dependent on *rev* co-expression (Fig. 3c lanes 1, 2), showing that the regulation of cytoplasmic HIV-1 RNA expression by Rev occurs independently from the regulation of HIV-1 RNA splicing.

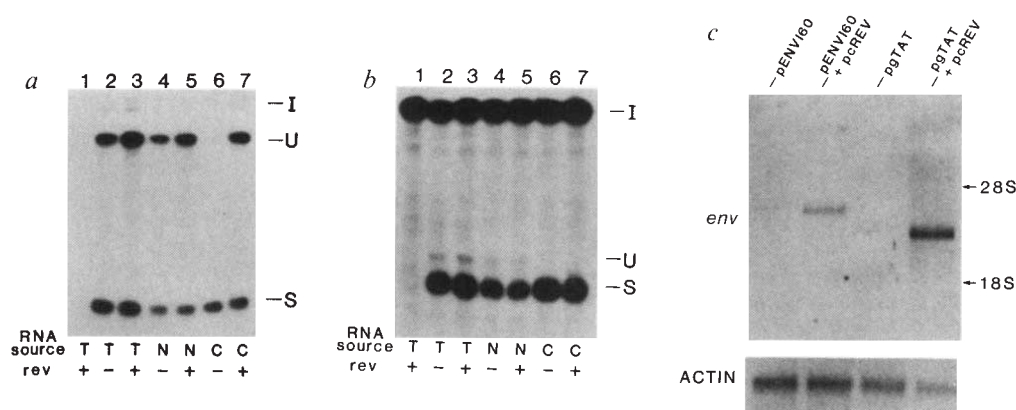
A major question raised by this study is why the high level of unspliced *tat* mRNA detected in the nucleus of transfected cells cannot enter the cytoplasmic compartment in the absence of Rev. One possibility is that the intronic sequences present in pgTAT repress unspliced RNA transport. Indeed, Rosen *et al.*¹⁴ have proposed the existence of four such *cis*-acting repressive (CRS) sequences within the HIV-1 *env* gene. In the absence of *rev* co-expression, the deletion mutant $\Delta K/Hd$, which lacks all four of these proposed repressive sequences, was, however, found to express the same phenotype as the parental pgTAT vector (Fig. 1c, lane 5). We therefore favour the alternative hypothesis that incompletely spliced HIV-1 mRNAs are prevented from leaving the nucleus by the same processes which preclude the export from the nucleus of many unspliced or incompletely spliced gene transcripts^{13,15-17}. The role of Rev would then be to permit incompletely spliced HIV-1 transcripts to interact with the nuclear RNA export machinery. It remains to be determined if the Rev *trans*-activator mediates the export of unspliced viral RNA from the nucleus by interacting directly with its RNA target sequence or whether Rev instead functions to post-translationally or transcriptionally activate cellular RNA sequence-recognition factors. □

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1. Feinberg, M. B., Jarrett, R. F., Aldovini, A., Gallo, R. C. & Wong-Staal, F. *Cell* **46**, 807-817 (1986).
2. Sodroski, J. *et al. Nature* **321**, 412-517 (1986).
3. Knight, D. M., Flomerfelt, F. A. & Ghayab, J. *Science* **236**, 837-840 (1987).

FIG. 3 The *rev* gene product activates the nuclear export of unspliced HIV-1 RNAs. a, S_1 nuclease analysis of spliced and unspliced *tat* gene RNA. COS cells were transfected⁶ with pgTAT and the genomic rat preproinsulin II gene expression vector pBC12M in the presence (+) or absence (-) of the *rev* gene expression vector pcREV⁶. Lane 1 contains RNA from a culture transfected with pcREV alone. Total (T) or fractionated cytoplasmic (C) and nuclear (N) RNA was prepared at 48 h after transfection as previously described (21, 22). 5 μ g of RNA was used for the total and cytoplasmic S_1 analyses while 2 μ g of RNA was used for the nuclear samples.

The input (I) probe is identical to the *tat* probe used in Fig. 1c, lanes 9-18 and allows the quantitation of both spliced (S) and unspliced (U) *tat* RNAs. The relative level of unspliced *tat* RNA in each sample was determined by densitometry using an LKB soft laser scanner and is: lane 2, 0.59; lane 3, 0.71; lane 4, 0.74; lane 5, 0.81; lane 6, 0.07; lane 7, 0.77. b, S_1 nuclease analysis of spliced and unspliced insulin gene RNA. The analysis visualized here was performed precisely as described for Fig. 3a, using the same RNA samples, except that the insulin-gene-specific S_1 probe described for Fig. 1c, lanes 1-4 was used. This input probe (I) permits the quantitation of both spliced (S) and unspliced (U) insulin RNA. c, Northern analysis²³ of cytoplasmic *env* gene mRNA. COS cell cultures were transfected⁶ with pgTAT or pENV160 in the presence or absence of pcREV. As the *env* gene in pENV160 is under



HIV-1 LTR control, the *tat* gene expression vector pcTAT⁶ was also included in each transfection. Cytoplasmic RNA was isolated^{21,22} at 48 h after transfection and 5- μ g aliquots used for each lane. The unspliced RNA encoded by pgTAT is predicted to be 3.0 kilobases (kb) in length, whereas the non-spliceable *env* mRNA encoded by pENV160 has a predicted size of 3.5kb^{3,6}. The filter was initially probed (upper panel) with an *env* gene specific probe derived from the *Bgl*II (B2) to *Hind*III (Hd) fragment shown in Fig. 1a. Subsequently, the filter was rehybridized with an actin probe as a loading control (lower panel).

4. Terwilliger, E. *et al.* *J. Virol.* **62**, 655–658 (1988).
5. Sadeie, M. R., Benter, T. & Wong-Staal, F. *Science* **239**, 910–914 (1988).
6. Malim, M. H., Hauber, J., Fenrick, R. & Cullen, B. R. *Nature* **335**, 181–183 (1988).
7. Gutman, D. & Goldenberg, C. J. *Science* **241**, 1492–1495 (1988).
8. Cullen, B. R. *Cell* **46**, 973–982 (1986).
9. Le, S.-Y., Chen, H.-H., Braun, M. J., Gonda, M. A. & Maizel, J. V. *Nucleic Acids Res.* **16**, 5153–5168 (1988).
10. Muesing, M. A., Smith, D. H. & Capon, D. J. *Cell* **48**, 691–701 (1987).
11. Feng, S. & Holland, E. C. *Nature* **334**, 165–167 (1988).
12. Katz, R. A., Kotler, M. & Skalka, A. M. *J. Virol.* **62**, 2686–2695 (1988).
13. Arrigo, S. & Beemon, K. *Molec. Cell Biol.* **8**, 4858–4867 (1988).
14. Rosen, C. A., Terwilliger, E., Dayton, A., Sodroski, J. G. & Haseltine, W. A. *Proc. natn. Acad. Sci. U.S.A.* **85**, 2071–2075 (1988).
15. Nevins, J. R. *A. Rev. Biochem.* **52**, 441–466 (1983).
16. Darnell, J. E. *Prog. Nucleic Acid Res. Molec. Biol.* **19**, 493–511 (1976).
17. Buchman, A. R. & Berg, P. *Molec. cell. Biol.* **8**, 4395–4405 (1988).
18. Ratner, L. *et al.* *Nature* **313**, 277–284 (1985).
19. Salsler, W. *Cold Spring Harb. Symp. quant. Biol.* **42**, 993–1004 (1977).
20. Cech, T. R. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **80**, 3903–3907 (1983).
21. Greenberg, M. E. & Ziff, E. B. *Nature* **311**, 433–438 (1984).
22. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. *Biochemistry* **18**, 5294–5304 (1979).
23. Thomas, P. S. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5201–5205 (1980).

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Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56^{lck}

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THE CD4 T-cell surface antigen is an integral membrane glycoprotein of relative molecular mass 55,000 which binds class II major histocompatibility complex (MHC) molecules expressed on antigen presenting cells (APCs). It is thought to stabilize physical interactions between T cells and APCs (for a review, see ref. 1). Evidence is accumulating that suggests that CD4 can transduce an independent signal during T-cell activation^{2–4}. It has recently been shown that CD4 expressed on human^{5,6} and murine⁶ T cells is physically associated with the Src-related tyrosine protein kinase p56^{lck} (refs 7, 8). These results indicate that CD4 can function as a signal transducer and suggest that tyrosine phosphorylation events may be important in CD4-mediated signalling. Here, we present evidence that cross-linking of the CD4 receptor induces a rapid increase in the tyrosine-specific protein kinase activity of p56^{lck} and is associated with the rapid phosphorylation of one of the subunits (ζ) of the T-cell receptor complex on tyrosine residues. These data provide direct evidence for a specific CD4 signal transduction pathway that is mediated through p56^{lck} and suggest that some of the tyrosine phosphorylation events detected during antigen-mediated T-cell activation may result from signalling through this surface molecule.

To test whether binding of ligand to the extracellular domain of CD4 results in an intracellular signal involving alterations of p56^{lck}, we examined the effects of antibody-mediated cross-linking of cell-surface CD4 on the enzymatic activity of p56^{lck} (Fig. 1). Cloned CD4⁺/CD8[−] murine T lymphocytes (C8, see Fig. 1 legend)⁹ were incubated with anti-CD4 monoclonal anti-

body (mAb) GK1.5 (which reacts against the extracellular domain of CD4) and rabbit anti-rat (RAR) immunoglobulin G. The effects of this treatment on the tyrosine kinase activity of p56^{lck} were analysed by immune-complex kinase assays using Lck-specific antisera (Fig. 1a). CD4 cross-linking resulted in a rapid (within 30 seconds) and significant (three to fivefold) increase in the tyrosine-specific protein kinase activity of p56^{lck}, as measured by *in vitro* autophosphorylation and phosphorylation of enolase, an exogenous substrate. A parallel Lck immunoblot (Fig. 1a, bottom panel) showed that this change is not attributable to an increase in the abundance of p56^{lck}, suggesting that it reflects an increase in 'specific' enzymatic activity. Similar results were obtained with another CD4⁺ murine T-cell clone (B10; ref. 6) but were not observed in the CD4[−] murine T-cell hybridoma 16M9 (ref. 10). The abundance of the Lck protein seemed to diminish after a few minutes of cross-linking (bottom panel, lanes 4 and 5), consistent with our previous observations⁶.

The phosphorylated species migrating at about 64,000 (64K) on this gel appears to result from a non-specific immunoprecipitation (our unpublished data). Indeed, using lysis conditions that preserve the enzymatic activity of Lck as well as its interaction with CD4, another protein, p64, can be recovered by a variety of polyclonal and monoclonal antisera (including pre-immune sera). Although prominent in the C8 cells, it is not detected in murine thymocytes in which a similar stimulation of p56^{lck} and enolase phosphorylation can be detected after CD4 cross-linking (manuscript in preparation). Preliminary data indicate that p64 may represent a contaminating serine kinase (unpublished data).

Further experiments (Fig. 1b) showed that the stimulation of Lck kinase activity depends on the physical cross-linking of CD4 as the enzymatic activity was unchanged by treatment with monovalent (antigen-binding) fragments (Fab) of mAb GK1.5 (Fig. 1b, lane 2). The small increase in kinase activity seen with mAb GK1.5 alone (lane 3) is probably a result of a small amount of CD4 cross-linking induced by the bivalent antibody or by aggregates of the monoclonal antibody. The specificity of the induction of Lck kinase activity by cell-surface antigen cross-linking was evaluated (Fig. 1c). Cross-linking of CD4 with a different anti-CD4 mAb (H129.19) (Fig. 1c, lane 3) also resulted in a rapid activation of p56^{lck}. Cross-linking of other surface molecules however, such as the T-cell antigen receptor (TCR) complex, which can induce T-cell activation (lane 4), Thy1.2 (lane 5) as well as cross-linking antibody directed against CD8 (using an antibody with the same isotype as mAb GK1.5) (lane 6) did not result in any alteration in enzymatic activity or abundance of the Lck protein.

Because not all p56^{lck} molecules expressed in CD4⁺ T cells are complexed with CD4 (ref. 6), a more accurate estimate of the degree of enzymatic activation of p56^{lck} requires a specific analysis of the population of Lck molecules associated with CD4. Immune-complex kinase assays of CD4 immunoprecipitates (Fig. 2a) revealed that CD4 cross-linking induced a six to eightfold increase in Lck kinase activity (Fig. 2a, lane 2). Additional experiments established that CD4 cross-linking did not significantly alter the enzymatic activity of the non-CD4 associated Lck kinase (Fig. 2b, lane 2).

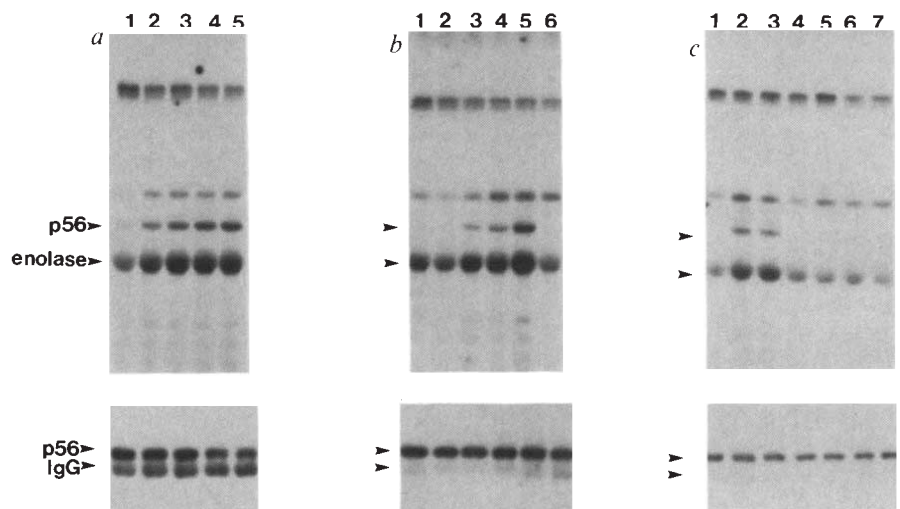
The only substrate of an activated tyrosine kinase detected during antigen presentation that has been defined is the ζ -subunit of the TCR complex, which is phosphorylated on tyrosine residues within minutes of antigen-mediated T-cell activation^{11–13}. The data reported here raise the possibility that signalling through the CD4-Lck pathway may participate in this phosphorylation event. To determine whether CD4 cross-linking could mediate this alteration, the effect of antibody-mediated cross-linking of CD4 on the state of tyrosine phosphorylation of the ζ -subunit of the TCR complex was examined (Fig. 3). After treating CD4⁺ murine T cells (C8) in various ways, the components of the TCR complex were immunoprecipitated with

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specific anti- ζ -antibodies and their phosphotyrosine content analysed by antiphosphotyrosine immunoblotting. This is a sensitive and specific method for evaluating tyrosine phosphorylation changes of this TCR complex component¹¹⁻¹³. The results of several experiments demonstrated that cross-linking of CD4 results in a three to fourfold increase in the phosphotyrosine content of the 21K phosphorylated form of the ζ -subunit (Fig. 3a, lane 3; data not shown). In these cells, the extent of the

detectable increase in ζ -subunit phosphotyrosine content was similar to that induced upon activation with the combination of a stimulatory antibody to the TCR complex (mAb 145-2c11) cross-linked by Fc receptor-bearing B cells (Fc is the crystallizable fragment of an immunoglobulin) (lane 5). A detailed analysis of these modifications showed that the increase in ζ -subunit tyrosine phosphorylation occurs as soon as one minute after CD4 cross-linking, reaching a maximum after 10-15 min (Fig.

FIG. 1 Effects of surface antigen cross-linking on p56^{lck}. **a**, Time course of the effects of antibody-mediated cross-linking of CD4 on p56^{lck} from a CD4⁺ murine T-cell clone (C8). Lane 1, untreated control; 2, 30 s; 3, 1 min; 4, 2 min and 5, 3 min. Top panel, autophosphorylation and enolase phosphorylation in *in vitro* kinase reactions. Bottom panel, Lck immunoblot assays. The positions of p56^{lck} (p56), enolase and IgG are indicated. The low level of autophosphorylation seen with Lck molecules from control cells (lane 1) is related to the presence of 10 μ M non-radioactive ATP in the enolase phosphorylation reactions, a situation which favours the phosphorylation of the exogenous substrate. Lck autophosphorylation at significant levels (also observed after CD4 cross-linking) can be observed in control cells when the assay mixture contains 1 μ M ATP. Similar observations have been made for the *c-src* tyrosine kinase (our unpublished data). Exposure times were: kinase, 6 h; immunoblot, 18 h. **b**, Effects of various treatments with anti-CD4 mAb (mAb GK1.5) on p56^{lck}. Cross-linking was performed for 1 min. Lane

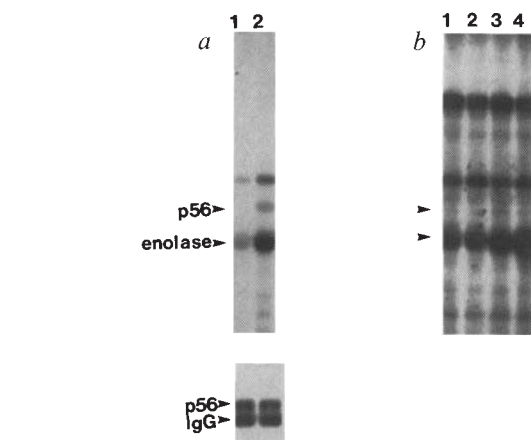


1, untreated control; 2, Fab fragments of mAb GK1.5; 3, mAb GK1.5; 4, mAb GK1.5 with RAR IgG added to the lysate; 5, mAb GK1.5 + RAR IgG and 6, RAR IgG. Top panel, kinase reactions (autophosphorylation and enolase phosphorylation). Bottom panel: Lck immunoblot. Exposure times were as in **a**. **c**, Effects of antibody-mediated cross-linking of various cell-surface antigens on p56^{lck}. Cross-linking was performed for 1 min (similar results were obtained after cross-linking for 5 min, data not shown). Lane 1, untreated control; 2, mAb GK1.5 + RAR IgG; 3, anti-CD4 mAb H129.19 + RAR IgG; 4, anti-CD3 mAb 145-2C11 + rabbit anti-hamster (RAH) IgG; 5, anti-Thy1.2 mAb 30-H12 + RAR IgG; 6, anti-CD8 mAb 2.43 + RAR IgG; and 7, RAR IgG. Top panel, kinase reactions (autophosphorylation and enolase phosphorylation). Bottom panel, Lck immunoblot. Exposure times were kinase, 6 h; immunoblot, 14 h. **METHODS.** C8 is an antigen-specific CD4⁺ murine T-cell hybridoma derived from B6 mice¹⁵. Cells were grown and prepared as described elsewhere^{6,15}. Resting C8 cells (10⁷ cells) were incubated on ice with saturating concentrations of various mAbs for 30 min, washed in cold PBS and incubated at room temperature in serum-free DMEM with or without RAR or RAH IgG (1.0 μ g; Organon Teknica) as second-step antibodies. After cross-linking, cells were lysed directly in DMEM by adding an equal volume of modified 2 $\times$ TNE buffer

(100 mM Tris pH 8.0; 2% NP-40 and 40 mM EDTA, pH 8.0) containing 200 μ M sodium orthovanadate, 100 mM sodium fluoride and the protease inhibitors leupeptin and aprotinin (each at a concentration of 20 μ g ml⁻¹). Similar results were obtained if the cells were extensively washed in cold PBS before lysis (data not shown). After clarification of the detergent lysate by centrifugation (10,000g for 5 min at 4 °C in an Eppendorf microfuge, this centrifugation does not alter the recovery of p56^{lck}, at least within the first few minutes of cross-linking (see Fig. 1)), Lck proteins were immunoprecipitated from equivalent amounts of cellular proteins (50 μ g) as described¹⁶. Immune-complex kinase assays were performed on washed immunoprecipitates in the presence of 10 μ M ATP and rabbit muscle enolase, the model substrate¹⁶. The reactions were stopped by the addition of sample buffer and the products of these reactions resolved by 8% SDS-PAGE¹⁶. The Lck immunoblot of parallel immunoprecipitates was performed as described⁸. All monoclonal antibodies have been previously described⁶ and were used as ammonium sulphate precipitation of ascites. Fab fragments of mAb GK1.5 were kindly provided by Ada Kruisbeek, NCI. We quantitated the changes described in the text by cutting bands from the gel or nitrocellulose and counting in a γ -counter or a liquid scintillation counter.

FIG. 2 Effects of CD4 cross-linking on the CD4-associated and non-CD4 associated p56^{lck}. **a**, Effect of CD4 cross-linking on activity of the CD4-associated Lck kinase. Lane 1 untreated control; and 2, mAb GK 1.5 + RAR IgG. Top panel, kinase assays; bottom panel, Lck immunoblot. The positions of p56^{lck}, enolase and IgG are indicated. Exposure times were: kinase, 6 hours; immunoblot, 18 hours (this experiment was done in parallel with that in Fig. 1a). **b**, Depletion experiments. Lanes 1 and 2, preclearing with anti-CD4 mAb GK 1.5; 3 and 4, preclearing with anti-Thy1.2 mAb 30-H12. Lanes 1 and 3, untreated cells; 2 and 4, after CD4 cross-linking. The restriction of Lck kinase induction to CD4-associated Lck molecules was demonstrated in a similar way by examining p56^{lck} autophosphorylation in the presence of 1 μ M ATP (our unpublished data). The high level of background seen in these assays seems to be related to the extensive manipulations of the cellular lysates. Exposure time was 24 h.

METHODS. C8 cells were treated with a CD4 cross-linking antibody regimen (mAb GK 1.5 + RAR IgG) for 1 min and lysed as described in the Fig. 1 legend. CD4 was immunoprecipitated from equivalent amounts of cellular proteins (50 μ g) by a combination of mAb GK 1.5, RAR IgG and *Staphylococcus aureus* protein A (this regimen was shown to recover all CD4-associated Lck, even after antibody-mediated cross-linking of surface CD4 with mAb GK 1.5 and RAR IgG, our unpublished data). For depletion experiments, the cellular lysate was first incubated at 4 °C for 2 h in the presence of the anti-CD4 mAb GK 1.5 or the anti-Thy1.2 mAb 30-H12 and then for 1 h in the presence of *Staph. aureus* protein A coupled to RAR IgG. After removing the immune



complexes by centrifugation, the residual Lck proteins in the supernatant were collected by the addition of Lck-specific antisera¹⁰. Immune-complex kinase assays and immunoblot were performed as previously described¹⁶.

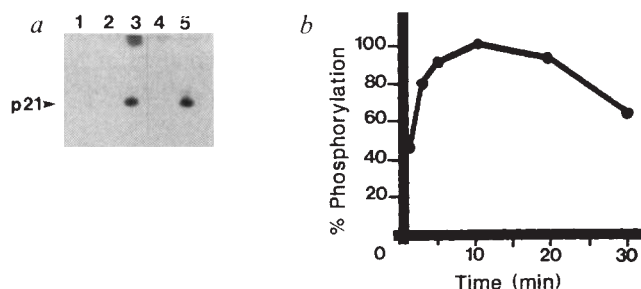


FIG. 3 Effect of CD4 cross-linking on the phosphorylation of ζ -subunit of the TCR complex. *a*, Effects of various antibody regimens. Lane 1, untreated control; 2, mAb GK1.5; 3, mAb GK1.5 and RAR IgG; 4, RAR IgG alone and 5, C8 cells incubated with the B-cell tumour LB and the anti-CD3 (epsilon) mAb 145-2C11 (ref. 6). Exposure was for 72 h. *b*, Time course. C8 cells were incubated with mAb GK1.5, washed and incubated at 37 °C as in Fig. 3a for the time indicated with RAR IgG. The level of ζ -chain phosphorylation was analysed with an LKB densitometer and is expressed here as a percentage of maximum- ζ -subunit phosphorylation. The results presented here are derived from the combined analysis of two separate experiments. In both cases, baseline p21(ζ) tyrosine phosphorylation was higher than that seen in the experiment represented in Fig. 3a. The basis for this variability is unclear. METHODS. C8 cells were subjected to CD4 cross-linking as described in Fig. 1 or cultured at a 1:1 ratio with the B-cell tumour LB 27.4 plus mAb 145-2C11 culture supernatant at 37 °C (used as T-cell activating stimuli). Cells were lysed as described and immunoprecipitated with an anti-peptide antibody recognizing the TCR- ζ -chain¹³. Electrophoresis and immunoblotting with affinity-purified anti-phosphotyrosine antibodies was performed as previously described¹¹⁻¹³. Immunoprecipitation of p21 was specific for the ζ -subunit (control sera failed to precipitate this structure; data not shown). The specificity of the anti-phosphotyrosine antibodies used in these assays has previously been described¹¹⁻¹³.

3b). Similar increases in ζ -subunit tyrosine phosphorylation were also detected after CD4 cross-linking of another CD4⁺ murine T-cell clone as well as a CD4⁺ T-cell tumour line, whereas no effect was seen upon similar treatment of a CD4⁻ T-cell hybridoma (L.E.S., unpublished data). It is unlikely that the tyrosine phosphorylation of the ζ -subunit reported here represents a non-specific reaction to cell-surface antigen cross-linking, as cross-linking of other surface molecules (such as LFA-1 and H-2) does not result in any significant alteration in the phosphorylation state of the ζ -subunit (L.E.S., unpublished data).

Our results indicate that the interaction of CD4 with its ligand results in a biochemical signal involving the rapid activation of p56^{lck}, providing direct evidence for the existence of a signalling pathway associated with CD4. These results also support the view that the CD4-Lck complex functionally mimics growth factor receptors with associated tyrosine kinase function. Although the exact mechanism of enzymatic activation of p56^{lck} remains to be established, preliminary data demonstrate that CD4 cross-linking is associated with a rapid and marked increase in the phosphorylation of Tyr 394 (major site of *in vitro* phosphorylation) and Tyr 505 (major site of *in vivo* phosphorylation) of p56^{lck} (our unpublished data). Interestingly, the phosphorylation state of the analogous residues of pp60^{c-src} has a significant effect on its tyrosine kinase activity (for a review, see ref. 14). □

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- Swain, S. *Immunol. Rev.* **74**, 129-142 (1983).
- Bank, I. & Chess, L. *J. exp. Med.* **162**, 1294-1303 (1985).
- Wassmer, P., Chan, C., Logdberg, L. & Shevach, E. *J. Immun.* **135**, 2237-2242 (1985).
- Rosoff, P. M., Burakoff, S. J. & Greenstein, J. L. *Cell* **49**, 845-853 (1987).
- Rudd, C. E., Trevillian, J. M., Dasgupta, J. D., Wong, L. L. & Schlossman, S. F. *Proc. natn. Acad. Sci. U.S.A.* **85**, 5190-5196 (1988).
- Veillette, A., Bookman, M. A., Horak, E. M. & Bolen, J. B. *Cell* **55**, 301-308 (1988).

- Marth, J. D., Peet, R., Krebs, E. G. & Perlmutter, R. M. *Cell* **43**, 393-404 (1985).
- Veillette, A., Foss, F. M., Sausville, E. A., Bolen, J. B. & Rosen, N. *Oncogene Res.* **1**, 357-374 (1987).
- Veillette, A., Horak, E. M., Horak, E. M., Bookman, M. A. & Bolen, J. B. *Molec. cell. Biol.* **8**, 4353-4361 (1988).
- Sleckman, B. P. *et al. J. Immun.* **141**, 49-54 (1988).
- Patel, M. D., Samelson, L. E. & Klausner, R. D. *J. biol. Chem.* **262**, 5831-5838 (1987).
- Bayanash, M., Garcia-Morales, P., Bonafacio, J. S., Samelson, L. E. & Klausner, R. D. *J. biol. Chem.* (in the press).
- Samelson, L. E. *et al. J. Immun.* **139**, 2708-2714 (1987).
- Veillette, A. & Bolen, J. B. in *Oncogenes* (Kluwer Academic, Norwell, Massachusetts, in the press).
- Bookman, M. A., Swerdlow, R. & Matis, L. A. *J. Immun.* **139**, 3166-3170 (1987).
- Veillette, A., Horak, E. M. & Bolen, J. B. *Oncogene Res.* **2**, 385-401 (1988).

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Association of dystrophin and an integral membrane glycoprotein

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DUCHENNE muscular dystrophy (DMD) is caused by a defective gene found on the X-chromosome¹. Dystrophin is encoded by the DMD gene and represents about 0.002% of total muscle protein². Immunochemical studies have shown that dystrophin is localized to the sarcolemma in normal muscle but is absent in muscle from DMD patients³⁻⁷. Many features of the predicted primary structure of dystrophin are shared with membrane cytoskeletal proteins⁸, but the precise function of dystrophin in muscle is unknown. Here we report the first isolation of dystrophin from digitonin-solubilized skeletal muscle membranes using wheat germ agglutinin (WGA)-Sepharose. We find that dystrophin is not a glycoprotein but binds to WGA-Sepharose because of its tight association with a WGA-binding glycoprotein. The association of dystrophin with this glycoprotein is disrupted by agents that dissociate cytoskeletal proteins from membranes. We conclude that dystrophin is linked to an integral membrane glycoprotein in the sarcolemma. Our results indicate that the function of dystrophin could be to link this glycoprotein to the underlying cytoskeleton and thus help either to preserve membrane stability or to keep the glycoprotein non-uniformly distributed in the sarcolemma.

The specific binding of dystrophin to wheat germ agglutinin (WGA)-Sepharose is shown in Fig. 1 (a-d). Dystrophin was solubilized from rabbit skeletal muscle membranes with 0.5 M NaCl and 1% digitonin (Fig. 1a and b, lane 1). The solubilized membranes were first passed through an antibody affinity column to remove the ryanodine receptor⁹ (relative molecular mass, M_r , 450,000). This step is important because of the similar apparent mobilities on SDS-polyacrylamide gels of dystrophin and a major proteolytic fragment of the ryanodine receptor⁹. The flow-through of the ryanodine-receptor affinity column was then applied to a WGA-Sepharose 4B column. Immunoblot-analysis of solubilized membranes and of solubilized membranes that had been repeatedly passed through a WGA-Sepharose column showed that dystrophin was completely removed from solubilized membranes by WGA-Sepharose (Fig. 1b, lane 2). Coomassie-blue staining of both samples run on SDS-polyacrylamide gels (Fig. 1a) indicated that there had been no change in the protein components because most of the proteins in the rabbit skeletal muscle membrane preparation are not WGA-binding glycoproteins. The protein adsorbed to WGA-Sepharose was also analysed by SDS-PAGE and immunoblotting (Fig. 1c and d). In the absence of *N*-acetyl-D-glucosamine (NAG), the α_1 and α_2 subunits of the dihydropyridine (DHP) receptor and a protein of M_r 400K were the main proteins bound to WGA-

Sephacrose (Fig. 1c, lane 1). The high-molecular weight protein was identified as dystrophin by immunoblotting (Fig. 1d, lane 1). The specificity of the absorption of dystrophin to WGA-Sepharose was tested by repeating the experiment in the presence of NAG. Only myosin ($M_r \approx 205K$) and the ($Ca^{2+} + Mg^{2+}$) ATPase ($M_r \approx 105K$) bound nonspecifically to WGA-Sepharose in the presence of NAG (Fig. 1c, lane 2). Our results therefore demonstrate that dystrophin specifically binds to WGA-Sepharose following solubilization in digitonin. The specific elution of dystrophin from WGA-Sepharose by NAG is shown in Fig. 1e and f; the yield of NAG-eluted material is ~ 5 mg from 800 mg starting membranes. The peak protein fractions that are eluted with NAG are enriched in dystrophin and the DHP receptor, as shown by Coomassie-blue staining and immunoblotting of SDS-polyacrylamide gels. Dystrophin makes up about 5% of the total NAG-eluted protein as estimated from integration of densitometer scans of the gels.

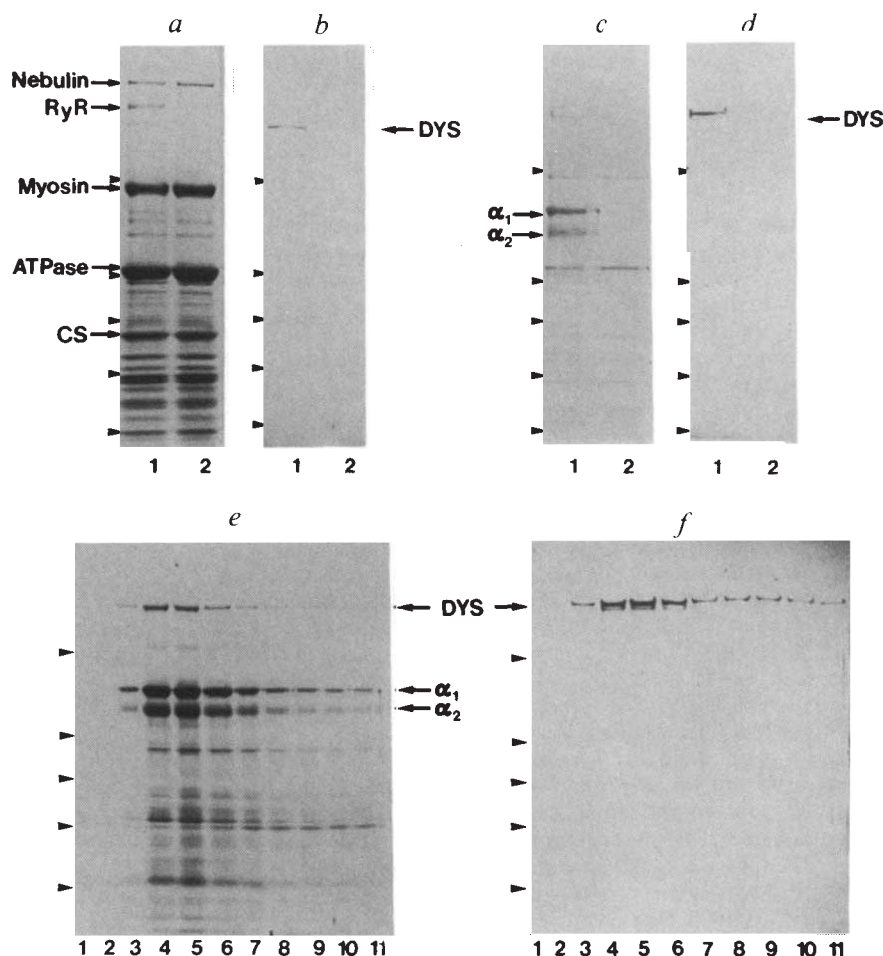
The fact that dystrophin could be isolated using WGA-Sepharose following detergent solubilization of skeletal muscle membranes indicated to us that dystrophin might be associated with the DHP receptor, or that it could be a glycoprotein. To investigate the possible association of dystrophin with the DHP receptor, we combined and fractionated NAG-eluted fractions from WGA-Sepharose using DEAE-cellulose. Elution of the DEAE-cellulose column with a linear gradient of NaCl indicated

that most of the DHP receptor eluted in a broad pattern from 50–110 mM NaCl, whereas dystrophin eluted at 110–300 mM NaCl. Consequently we developed a step gradient of NaCl to separate the DHP receptor from dystrophin more effectively (Fig. 2). The first four fractions are enriched in DHP receptor and do not contain dystrophin (as determined by Coomassie blue-stained gels and immunoblots; Fig. 2a and b, lanes 2–5). The final fraction is enriched in dystrophin (corresponding to 30–40% of the protein by densitometry) and is essentially depleted of DHP receptor (Fig. 2a and b, lane 6). Proteins co-eluting with dystrophin migrated on Coomassie blue-stained gels at positions corresponding to M_r s of 325K, 250K, 225K, 172K, 162K, 143K, 57K, 47K, 39K and 31K; the mobility of dystrophin indicated that its M_r was $\sim 360K$. Immunoblotting after each step in the preparation showed that dystrophin was isolated intact and not degraded. Thus the decrease in apparent M_r of isolated dystrophin when compared with the value of 427K predicted from the complementary DNA sequence⁸ may result from proteolytic processing after synthesis or simply from the difficulty in measuring high molecular weight proteins accurately on SDS gels.

To investigate whether dystrophin is a glycoprotein, we stained protein blots of DEAE-isolated dystrophin and purified DHP receptor with ^{125}I -labelled lectins. ^{125}I -labelled WGA specifically stained the α_2 subunit of the DHP receptor (Fig. 3,

FIG. 1 Specific binding of dystrophin to WGA-Sepharose (a–d) and elution with *N*-acetyl-D-glucosamine (e–f). Coomassie blue-stained gels (a, c and e) and immunoblots of identical gels stained with sheep polyclonal anti-dystrophin antibodies (b, d and f) are shown. a and b, Lane 1, digitonin-solubilized rabbit skeletal-muscle membranes (75 μ g); lane 2, column flow-through from four independent WGA-Sepharose columns (75 μ g). Arrows indicate the positions of nebulin, $\sim 700K$; ryanodine receptor/ Ca^{2+} -release channel, $\sim 450K$ (RyR); myosin, 205K; ($Ca^{2+} + Mg^{2+}$)ATPase, 105K (ATPase); and calsequestrin, 63K (CS). c and d, WGA-Sepharose beads (50 μ l) incubated with solubilized membranes in the absence (lane 1) or presence (lane 2) of *N*-acetyl-D-glucosamine. e and f, Lanes 1–11 are 100 μ l aliquots of NAG-eluted WGA-Sepharose column fractions. Arrows indicate the positions of dystrophin (DYS) in panels b, d, e and f and the α_1 and α_2 subunits of the DHP receptor in panels c and e. For all panels, arrowheads indicate the positions of the molecular weight standards: from top to bottom their M_r values ($\times 10^{-3}$) are 208, 100, 68, 43 and 25.

METHODS. For a and b, rabbit skeletal muscle membranes⁹ (800 mg) were solubilized with a solution of 1% digitonin and 0.5 M NaCl containing protease inhibitors at a protein concentration of 4 mg ml⁻¹, as previously described⁹, then passed through XA7-Sepharose for removal of ryanodine receptor⁹ and through four different columns of WGA-Sepharose (15 ml each). For c and d, membranes (2 mg) were solubilized and passed through XA7-Sepharose as already described, then applied to 50 μ l WGA-Sepharose in the absence or presence of 0.3 M NAG. After incubation the WGA-Sepharose beads were washed extensively and subjected to SDS-PAGE. For e and f, digitonin-solubilized membranes (800 mg) were applied to XA7-Sepharose and circulated overnight on 15 ml WGA-Sepharose. After extensive washing, the WGA-Sepharose column was eluted with five column volumes of 0.3 M NAG and 2.5 ml fractions were collected. Yields of 3 to 5 mg from 800 mg solubilized membranes were typical. In all experiments, WGA-Sepharose buffers contained 50 mM Tris-HCl, pH 7.4, 0.75 mM benzamidine and 0.1 mM phenylmethylsulphonyl fluoride. Samples were fractionated on 3–12% gradient SDS-polyacrylamide gels¹⁷



and either stained with Coomassie blue or transferred to nitrocellulose paper¹⁸ and stained with either sheep polyclonal anti-dystrophin antibodies¹⁹ or anti- α_1 and anti- α_2 antibodies^{20,21} for identification of the α_1 and α_2 subunits of the DHP receptor (not shown). Gel lanes were scanned using a Hoefer GS 300 scanning densitometer and analysed using GS-360 data analysis software. Protein concentrations were determined as previously described²².

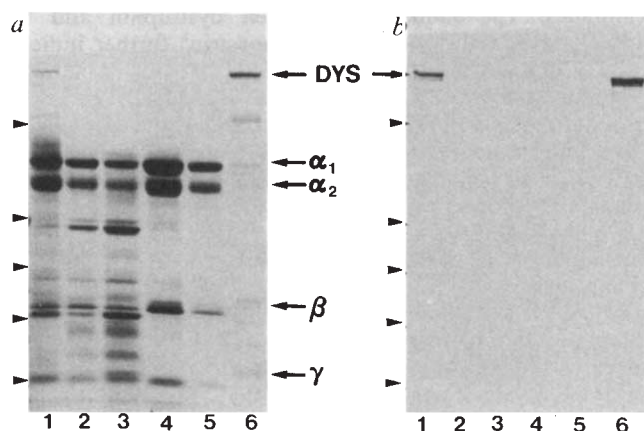


FIG. 2 Isolation of dystrophin using DEAE-cellulose. A Coomassie blue-stained gel (a) and an immunoblot of an identical gel stained with sheep polyclonal anti-dystrophin antibodies (b) are shown. Lane 1, 10 μ g NAG-eluted sample (Fig. 1e, lane 5); lane 2, 13 μ g fraction 1 from the 50 mM NaCl wash from the DEAE column; lane 3, 15 μ g fraction 2 from the DEAE-column 50 mM NaCl wash; lane 4, 25 μ g fraction 1 from the DEAE-column 100 mM NaCl wash; lane 5, 11 μ g fraction 2 from the DEAE-column 100 mM NaCl wash; lane 6, 8 μ g fraction 1 from the DEAE-column 250 mM NaCl wash. Arrows indicate the positions of dystrophin (DYS) and the α_1 , α_2 , β and γ subunits of the DHP receptor. Molecular weight standards (arrowheads) are the same as those used for Fig. 1.

METHODS. Eluted fractions from WGA-Sepharose (Fig. 1e) were applied to 2 ml packed DEAE-Cellulose equilibrated in buffer A (0.1% digitonin, 50 mM Tris-HCl, pH 7.4, 0.75 mM benzamidine and 0.1 mM phenylmethylsulphonyl fluoride). The DEAE-column was washed with 10 ml buffer A, followed by buffer A containing 50 mM NaCl (50 ml), 100 mM NaCl (200 ml), 110 mM NaCl (100 ml) and 250 mM NaCl (25 ml). Fractions (2.5 ml) were collected for all washes. Analysis of the 110 mM NaCl wash indicated a protein pattern similar to that in lanes 4 and 5, but much less intense (not shown). Washing of the DEAE-column with NaCl concentrations between 110 mM NaCl and 250 mM NaCl revealed a protein pattern similar to that in lane 6 and did not preferentially remove any protein (not shown). Approximately 300 μ g isolated dystrophin (lane 6) was obtained from 700 mg solubilized membranes. SDS-PAGE, immunoblot analysis and densitometer scanning are described in the legend to Fig. 1.

lane 1) but did not stain dystrophin (Fig. 3, lane 2). Concanavalin A (con A) labelled with 125 I was also used to stain blots and together with results of endoglycosidase H digests confirmed that dystrophin is not a glycoprotein (data not shown). So we conclude that dystrophin must be retained by WGA-Sepharose as a result of an association with a glyco-mediator which survives solubilization of the membranes. The isolated dystrophin also binds specifically to con A-Sepharose (not shown). As glycoproteins are the only molecules that will specifically bind both to WGA-Sepharose and con A-Sepharose, the mediator must be a glycoprotein. We also show (Fig. 3) that the dystrophin preparation contains several glycoproteins ranging from 40K to 500K, but no glycolipids. Therefore the mediator of the interaction between dystrophin and the lectin columns must be a glycoprotein containing both WGA and con A binding sites. Also, as the detergent concentration necessary to solubilize dystrophin is almost identical to that required to solubilize the ryanodine and DHP receptors (both integral membrane proteins), then the mediator must also be an integral membrane glycoprotein.

To investigate this association of dystrophin with the integral membrane glycoprotein, we attempted to dissociate the dystrophin-glycoprotein complex with treatments known to disrupt the interaction between cytoskeletal proteins and membranes¹⁰⁻¹², and then monitored the binding of dystrophin to

WGA-Sepharose. We used NAG-eluted dystrophin because we needed large amounts of protein for Coomassie-blue staining and because it contained the DHP receptor, which is a complex of several polypeptides with a major glycoprotein¹³. Figure 4 shows that NAG-eluted dystrophin rebinds completely to WGA-Sepharose after removal of NAG (lane 1). Thus, dystrophin retains its association with the WGA-binding glycoprotein after isolation and dialysis to remove NAG. When the NAG-eluted sample is treated with 1% SDS, followed by dilution to 0.1% SDS and removal of NAG by dialysis, dystrophin no longer binds to WGA-Sepharose (Fig. 4a and b, lane 2), even though the α_2 subunit of the DHP receptor (a WGA-binding glycoprotein) still binds to WGA-Sepharose (Fig. 4c and d, lane 2). Thus, SDS is able to dissociate the dystrophin-glycoprotein complex and the free dystrophin does not bind to WGA-Sepharose.

Membrane cytoskeletal protein interactions are known to be sensitive to molar concentrations of potassium iodide^{10,11}. We tested the effect of KI on the interaction of dystrophin with the WGA-binding glycoprotein by treating the NAG-eluted sample with 2 M KI. After dilution of KI to 0.5 M and removal of NAG by dialysis, the sample was applied to WGA-Sepharose. In Fig. 4, lane 3 it is shown that KI dissociates the dystrophin-glycoprotein complex and that KI-treated dystrophin no longer binds to WGA-Sepharose, even though the α_2 subunit of the

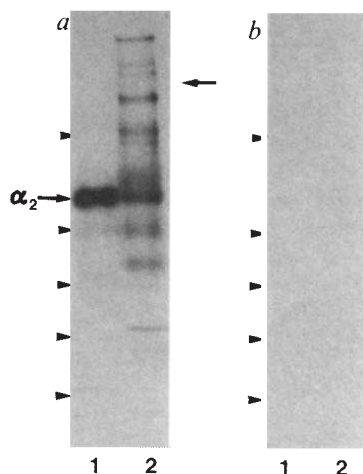


FIG. 3 Glycoprotein analysis of purified DHP receptor and isolated dystrophin using 125 I-labelled WGA. Autoradiographs of immunoblots incubated with 125 I-labelled WGA in the absence (a) or presence (b) of 0.3 M NAG are shown. Lane 1, 7 μ g purified DHP receptor (Fig. 2, lane 4); lane 2, 7 μ g isolated dystrophin (Fig. 2, lane 6). The position of the α_2 subunit of the DHP receptor is indicated by the arrow (α_2). The arrow on the right in panel a indicates the position of dystrophin determined by staining the blot with sheep polyclonal anti-dystrophin antibodies (after staining with 125 I-labelled WGA) and then overlaying the autoradiograph on the blot. Note that dystrophin does not stain with 125 I-labelled WGA. Molecular weight standards (arrowheads) are the same as those used for Fig. 1.

METHODS. DHP receptor (Fig. 2, lane 4) or isolated dystrophin (Fig. 2, lane 6) were separated on 3-12% SDS-polyacrylamide gels and transferred to nitrocellulose paper. Blots were blocked in PBS-Tween (50 mM NaH₂PO₄, 0.9% NaCl, 0.05% Tween-20) and incubated for 1 h at 25 °C with 250,000 c.p.m. 125 I-labelled WGA per ml PBS-Tween either with or without 0.3 M NAG. Transfers were washed extensively with PBS-Tween then with PBS, then dried and exposed to Kodak-XAR film using intensifying screens (Dupont). Exposures were for three days at 25 °C. No additional bands appeared after longer exposure. Identical results were obtained using 125 I-labelled con A (not shown). SDS-PAGE and immunoblot analysis are described in the legend to Fig. 1.

DHP receptor still binds to WGA-Sepharose. Similar results were obtained with con A-Sepharose (not shown). 125 I-labelled WGA staining of the material in the void volume from the WGA-Sepharose beads and of the NAG-eluted material (not shown) indicates that all WGA-binding glycoproteins bound to WGA-Sepharose after treatment with SDS or KI. So, dystrophin binds to WGA-Sepharose as a result of its tight association with a WGA-binding glycoprotein and this association is sensitive to SDS and KI.

Here we have reported the first isolation of dystrophin from skeletal muscle membranes: as dystrophin is present in very low concentrations in muscle, a procedure representing a 17,000-fold purification of dystrophin from whole muscle is an important advance. Previously dystrophin could only be studied indirectly, for example by using immunological probes for the protein. We have also demonstrated that dystrophin is tightly associated with

an integral membrane glycoprotein and that this association is disrupted by agents that dissociate cytoskeletal proteins from membranes. The similarities between dystrophin and the cytoskeletal proteins α -actinin and spectrin⁸ further indicate that the function of dystrophin could be to link an integral membrane glycoprotein to the underlying cytoskeleton. This association of dystrophin with a WGA-binding glycoprotein has been found in membranes from rabbit and rat cardiac muscle and skeletal muscle from guinea pig, dog, mouse and human (results not shown). We conclude that the localization of dystrophin to the cytoplasmic face of the sarcolemma results from a tight association with an integral sarcolemma membrane glycoprotein and we propose that this glycoprotein serves as a 'dystrophin receptor' which links dystrophin to the sarcolemma membrane. Although we have not yet identified this 'receptor', the results shown in Fig. 3 indicate that it could be one of the glycoproteins migrating with M_r 480K, 400K, 362K, 300K, 226K, 205K, 133K, 100K, 75K, 62K, 47K or 37K.

The dystrophin-glycoprotein complex may have an important structural and/or functional role in muscle: it could contribute to stabilization of the membrane or of a non-uniform distribution of a membrane glycoprotein, or it could link dystrophin to the extracellular matrix. We believe that the low concentration of dystrophin itself favours a role in maintaining the heterogeneous membrane arrangement of a scarce glycoprotein, possibly an ion channel or cell-surface receptor. The association of dystrophin with a sarcolemma glycoprotein is comparable to the association of ankyrin with red-cell or brain membranes¹⁰⁻¹² in that molar concentrations of KI can disrupt the complex. Recently ankyrin has been shown to be associated with the $(Na^+ + K^+)ATPase$ in kidney¹⁴ and with the Na^+ channel in the brain¹⁵; these associations with ankyrin were proposed to be responsible for the non-uniform distribution of these proteins in epithelial cells or neuronal cells respectively. Dystrophin may have a similar role in muscle and could be responsible for a non-uniform distribution of a membrane glycoprotein in the sarcolemma membrane.

The absence of dystrophin in DMD muscle may result in the displacement or dysfunction of the membrane glycoprotein that is normally associated with dystrophin. The lack of the dystrophin-glycoprotein complex may weaken sarcolemma membrane integrity and this may be critical for fast muscle fibres, which appear to be the first site of degeneration¹⁶. It remains to identify the 'dystrophin receptor' in the sarcolemma membrane and to determine the role of dystrophin in the function of the membrane glycoprotein. □

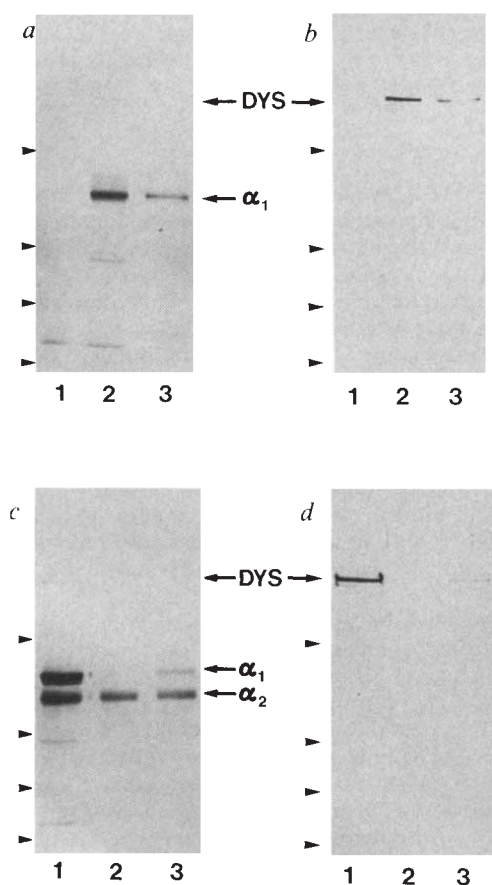


FIG. 4 Dissociation of dystrophin and membrane-associated glycoproteins using SDS and KI. Shown are Coomassie Blue-stained gels (a and c) and immunoblots of identical gels stained with sheep polyclonal anti-dystrophin antibodies (b and d). Lane 1, no treatment; lane 2, 1% SDS treatment; lane 3, 2 M KI treatment. In a and b, supernatants (100 μ l) from the WGA-Sepharose beads are analysed. In c and d, NAG-eluates (100 μ l) from the WGA-Sepharose beads used in a and b are analysed. The positions of dystrophin (DYS) and the α_1 and α_2 (c and d only) subunits of the DHP receptor are indicated by arrows. Molecular weight standards (arrowheads) are the same as those used for Fig. 1.

METHODS. 100 μ l (20 μ g) NAG-eluted sample from WGA-Sepharose (Fig. 1e, lane 5) was treated for 30 min at 25 $^{\circ}C$ either alone or with 1% SDS or 2 M KI. Samples were dialysed overnight in 50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.75 mM benzimidazole and 0.1 mM phenylmethylsulphonyl fluoride-containing buffer only for the no-treatment sample, 1% SDS for the SDS-treated sample, or 0.5 M KI for the KI-treated sample, and then applied to 50 μ l WGA-Sepharose prewashed either with buffer only, 0.1% SDS or 0.5 M KI. The samples were incubated for 2 h at 25 $^{\circ}C$ with agitation, then pelleted. Following extensive washing with the appropriate buffer (buffer only, 0.1% SDS or 0.5 M KI), the WGA-Sepharose beads were eluted with 0.3 M NAG. SDS-PAGE and immunoblot analysis are described in the legend to Fig. 1.

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- Walton, J. N. & Gardner-Medwin, D. in *Disorders of Voluntary Muscle* (ed. Walton, S. J.) 481-524 (Churchill Livingstone, Edinburgh, 1981).
- Hoffman, E. P., Brown, R. H. & Kunkel, L. M. *Cell* **51**, 919-928 (1987).
- Zubrzycka-Gaarn, E. E. et al. *Nature* **333**, 466-469 (1988).
- Arahata, K. et al. *Nature* **333**, 861-863 (1988).
- Watkins, S. C., Hoffman, E. P., Slayter, H. S. & Kunkel, L. M. *Nature* **333**, 863-866 (1988).
- Hoffman, E. P. et al. *New Engl. J. Med.* **318**, 1363-1368 (1988).
- Bonilla, E. et al. *Cell* **54**, 447-452 (1988).
- Koenig, M., Monaco, A. P. & Kunkel, L. M. *Cell* **53**, 219-228 (1988).
- Imagawa, T., Smith, J. S., Coronado, R. & Campbell, K. P. *J. Biol. Chem.* **263**, 16636-16643 (1987).
- Korsgren, C. & Cohen, C. M. *J. Biol. Chem.* **261**, 5536-5543 (1986).
- Bennett, V., Baines, A. J. & Davis, J. *Meth. Enzym.* **134**, 55-69 (1986).
- Ezzel, R. M., Kenney, D. M., Egan, S., Stossel, T. P. & Hartwig, J. H. *J. Biol. Chem.* **263**, 13303-13309 (1988).
- Campbell, K. P., Leung, A. T. & Sharp, A. H. *Trends Neurosci.* **11**, 425-430 (1988).
- Nelson, W. J. & Veshnock, P. J. *Nature* **328**, 533-536 (1987).
- Srinivasan, Y., Elmer, J., Bennett, V. & Angelides, K. *Nature* **333**, 177-180 (1988).
- Webster, C., Silberstein, L., Hayes, A. D. & Blau, H. M. *Cell* **52**, 503-513 (1988).
- Laemmli, U. K. *Nature* **227**, 680-685 (1970).
- Towbin, H., Staehelin, T. & Bordon, J. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4350-4354 (1979).
- Knudson, C. M., Hoffman, E. P., Kahl, S. D., Kunkel, L. M. & Campbell, K. P. *J. Biol. Chem.* **263**, 8480-8484 (1988).
- Leung, A. T., Imagawa, T. & Campbell, K. P. *J. Biol. Chem.* **262**, 7943-7946 (1987).
- Sharp, A. H. & Campbell, K. P. *J. Biol. Chem.* **264**, 2816-2825 (1989).
- Leung, A. T., Imagawa, T., Block, B., Franzini-Armstrong, C. & Campbell, K. P. *J. Biol. Chem.* **263**, 994-1001 (1987).

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A *Drosophila* tissue polarity locus encodes a protein containing seven potential transmembrane domains

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THE function of the *frizzled* (*fz*) locus in *Drosophila melanogaster* is required to coordinate the cytoskeletons of epidermal cells to produce a parallel array of cuticular hairs and bristles (for example on the wild-type wing all hairs point towards the distal tip)^{1,2}. In *fz* mutants it is not the structure of individual hairs and bristles that is altered, but their orientation with respect to their neighbours and the organism as a whole. Mitotic clone analysis³ indicates that *fz* has two functions in the developing wing. It is required for the proximal-distal transmission of an intercellular polarity signal, a process that is expected to be at least partly extracellular. It is also required for cells to respond to the polarity signal, which is expected to be a cytoplasmic function. The *fz* locus could encode either one bifunctional or two single-function proteins. We report here that, in pupae, *fz* produces a messenger RNA that encodes a protein with seven putative transmembrane domains. Thus, the Fz protein should contain both extracellular and cytoplasmic domains, which could function in the transmission and interpretation of polarity information, respectively. This is the first reported sequence for the protein product of a tissue polarity gene.

The *fz* locus was cloned by transposon tagging^{4,5} and *fz* complementary DNA clones isolated⁶ (the detailed molecular analysis of the *fz* locus will be presented elsewhere). A single 4 kilobase (kb) hybridization band is seen when northern blots of poly(A)⁺ pupal RNA are probed with cDNA sequences, suggesting that *fz* encodes a single bifunctional protein.

The 2,466-base pair (bp) sequence of a *fz* cDNA clone is shown in Fig. 1. There is a single complete long open reading frame of 581 amino acids. The Fz protein sequence has several interesting features. Most notably, hydrophobicity analysis^{7,8} reveals seven strongly hydrophobic stretches (regions 1 to 7 in Fig. 2), which are typical of transmembrane domains of integral membrane proteins. As *fz* is required for the transmission of an intercellular polarity signal, we suggest that the protein product is located in the plasma membrane of epidermal cells. The N terminus of the Fz protein probably encodes a signal sequence for membrane transport, as typical *n* (positively charged N-terminal, Arg 3), *h* (hydrophobic, Ile 5–Ile 13), and *c* (polar C-terminal, Gln 14–Asp 25) regions are present⁹. In addition, a potential cleavage site for the signal peptidase is present at Ala 26. This suggests that the terminus of Fz is extracellular. If each of the seven hydrophobic regions (Fig. 2) are trans-

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GAA TTC GAC TCC GTG TTT GTT CAG TTA TTT TTT TGC GCG GTT TTC GAA AAA GTG AAA GAA 60
ATA AGG AAA TTC AAA AAG CGG GGG AGG CAT AAA TGA TAT ACA GCT AAC GGT ATT TAT GTG 120
CAT ACA GCA ACA TAA AAA TGT GGC TGC TAG AAA AGT AAA GCC CGG TAT TTT GCC GCA TAA 180
AAG CCA ACG CCA CTA AGT CCT AAA GAC TCC CAA AAA TTA AAT TTT GAC ACA ATA TTT 240
TGC CAA CAA CAA ATA GCA AAG TCG AAT TCA AAC GGT TAA ACA AAA TTA AGC CCT GAA AAT 300
AAC AAA AAG GTC TAA ACA AAA TAA AGA GCA AAA GCA AAC GCA AAT AAG AGA ATA CAG AGA 360
AAT ATT TAT TAT TTG CTT ATA AGG CCA AAC TAA ATA TTT GCC GCC GGC AAA TGT TTA AAA 420
GGT GTT AAG TGT TTT GTC GGA GGG AAG TTT TTA AAC AGG AAG AAA AAT AAA TCA AAT AAT 480
CTA ATG GTG TAT AAG TGA GTG CAA GTA GAT GAG CAA CAT GTG TGT GTT TTA AAG TGT TTA 540
AAG AGA TCG AGA AAA AGC CCC AAA AGT TCT TGT ACA GCG ATA TCG AAA AAT TCC AAA ATG 600
Met 1

TGG CGT CAA ATC CTG TTT ATT TTA CCC ACC CTG ATA CAG GGG GTC CAG CGC TAC GAT CAG 660
Trp Arg Gln Ile Leu Phe Ile Leu Pro Thr Thr Leu Ile Gln Gly Val Gln Arg Tyr Asp Cys 720

AGC CCC CTC GAT GCG AGT GCG TAT TAT CGC AGC GGC GGG GGA TTA ATG GCC AGT TCT GGG 720
Ser Pro Leu Asp Ala Ser Pro Tyr Tyr Arg Ser Gly Gly Gly Leu Met Ala Ser Ser Gly 780

ACC GAA CTA GAT GGA CTG CCA CAT CAC AAT CGC TGT GAA CCC ATC ACC ATA TCG ATC TGC 780
Trp Glu Asp Gly Leu Pro His His Asn Arg Cys Gly Glu Gly Leu Thr Ile Ser Thr Cys 840

AAG AAT ATA CCA TAT AAC ATG ACC ATT ATG CCA AAT CTT ATT GGC CAT ACC AAG CAG GAG 840
Lys Asn Ile Pro Tyr Asn Met Thr Ile Met Pro Asn Leu Ile Gly His Thr Lys Gln Glu 900

GAG GCG GGT CTG GAG GTC CAT CAG TTT GCT CGC CTC GTG AAG ATC GGC TGC AGT GAT CAG 900
Glu Ala Gly Leu Asp Gly Val His Gln Phe Val His Gln Phe Val His Gln Phe Val His Gln 960

CTC CAG TTG TTC CTC TGT TCC CTG TAC GTT CCG GTC TGC ACA ATT TTG GAG CGA CCC ATT 960
Leu Gln Leu Phe Leu Cys Ser Leu Tyr Val Pro Val Cys Thr Ile Leu Glu Arg Pro Ile 1020

CCG CCT TGT CGA TCT CTG TGC GAA TCG GCG CGG GTA TGT GAA AAG TTA ATG AAA ACC TAC 1020
Pro Pro Cys Arg Ser Leu Cys Glu Ser Ala Arg Cys Glu Lys Leu Met Lys Thr Tyr 1080

AAC TTT AAC TGG CCG GAA AAT CTG GAG TGC TCC AAA TTT CCC GTC CAT GGA GGC GAG GAT 1080
Asn Phe Asn Trp Pro Glu Asn Leu Glu Cys Ser Lys Phe Pro Val His Gly Gly Glu Asp 1140

TTG TGC GTG GCG GAG AAT ACC ACA TCA TCG GCC TCC ACG GCG GCC ACG CCC ACA AGG AGT 1140
Leu Cys Val Ala Glu Asn Thr Thr Ser Ser Ala Arg Val Glu Ala Thr Pro Leu Ser Thr 1200

GTG GGT AAG GTC ACT ACC CGT AAA CAC CAG ACG GGC GTA GAA AGT CCG CAC GCA AAC ATT 1200
Val Ala Lys Val Thr Thr Arg Lys His Gln Thr Gly Val Glu Ser Pro His Arg Asn Ile 1260

GGA TTC GTG TGC CCC GTG CAA CTG AAA ACG CCG CTG GGA ATG GGC TAC GAA CTA AAA GTT 1260
Gly Phe Val Cys Pro Val Gln Leu Lys Thr Pro Leu Gly Met Gly Tyr Glu Leu Lys Val 1320

GGC GGA AAG GAT CTG CAT GAG TGT GGA GCC CCA TGT CAT GCC ATG TTC TGC GCG GAG AGA 1320
Gly Gly Lys Asp Leu His Asp Cys Gly Ala Pro Cys His Ala Met Phe Phe Pro Glu Arg 1380

GAA AGG ACT GTT CTT CCA TGA TGG GTT GGA TCC TGG GCA GCG GTC TGT GTT GCC AGC TGC 1380
Glu Arg Thr Val Leu Arg Tyr Trp Val Gly Ser Trp Ala Ala Val Cys Val Ala Ser Cys 1440

TTG TTT ACG GTG CTC ACC TTC TTG ATT CAG TCG TCG GTT TTT CCG TAC CCG GAG AGG GCC 1440
Leu Phe Thr Val Leu Thr Phe Leu Ile Asp Ser Ser Arg Phe Arg Tyr Pro Glu Arg Arg 1500

ATT GTC TTC TTG GCC GTT TGC TAC TTG GTA GTT GGA TGT GCC TAC GTG GCG GGA CTG GGG 1500
Ile Val Phe Leu Ala Val Cys Tyr Leu Val Cys Tyr Val Cys Tyr Val Ala Gly Leu Gly 1560

GCG GCG GAC TCT GTG TCG TGC GCG GAA CCA TTT CCG CCG CCC GTC CCA CTC GCG GCG CTC 1560
Ala Gly Asp Ser Val Ser Cys Arg Glu Pro Phe Pro Pro Val Lys Leu Gly Arg Leu 1620

CAG ATG ATG TCC ACC ATC ACC CAG GGC CAC GCA CAA ACC ACG TCC TGC AGC GTT TTA TTC 1620
Gln Met Met Ser Thr Ile Thr Gln Gly His Arg Gln Thr Thr Ser Cys Thr Val Leu Phe 1680

ATG GCA CTC TAC TCT TGT TGC ATG GCG GCG TTC GCG TGG TGC TGT CTG GCA CTC GCG 1680
Met Ala Leu Tyr Phe Cys Cys Met Ala Ala Phe Ala Trp Trp Ser Cys Leu Ala Phe Ala 1740

TGG TTT TTG GCT GCT GGC CTC AAA TGG GGC CAC GAG GCG ATT GAG AAC AAG TCG CAC TTA 1740
Trp Phe Leu Ala Ala Gly Leu Lys Trp Gly His Glu Ala Ile Glu Asn Lys Ser His Leu 1800

TTC CAC GTT GCT GCG TGG GCG GTG CCC GCG CTT CAG ACC ATT TCC GTT GTC GCG CTC GCT 1800
Phe His Leu Val Ala Trp Ala Val Pro Ala Val Pro Ala Leu Gln Thr Ile Ser Val Leu Leu Ala 1860

AAA GTT GAA GGT GAC ATC CTT TCT GGC GTT TGT TTC GTG GGT CAG CTG GAC ACC CAG TCC 1860
Lys Val Glu Gly Asp Ile Leu Ser Gly Val Cys Phe Val Gly Gln Leu Asp Thr His Ser 1920

CTG GCG GCG TTT CTG ATC CTT CCA CTC TGC ATT TAT CTC TCG ATT GGA GCA CTA TTC CTG 1920
Leu Gly Ala Phe Leu Ile Leu Pro Leu Cys Ile Tyr Leu Ser Ile Gly Leu Ala Leu Leu 1980

CTG GCG GGA TTT ATT TCG CTT TTC CCG ATC CCG ACA GTT ATG AAA ACG GAG GGA AAG AGG 1980
Leu Ala Gly Phe Ile Ser Leu Phe Arg Ile Arg Thr Val Met Lys Thr Asp Gly Lys Arg 2040

ACA GAC AAA CTG GAG GCG CTG ATG TTG CGA ATA GGT TTC TCT TCC GGA CTG TTC ATT CTG 2040
Thr Asp Lys Leu Glu Arg Leu Met Leu Arg Ile Gly Phe Phe Ser Gly Leu Phe Leu Ser 2100

CCC GCG GTG GGA TTA CTG GCG TGC TTG TTC TAC GAG TAC TAC AAC TTT GAG CAG TGG ATG 2100
Pro Ala Val Gly Leu Leu Gly Cys Leu Phe Tyr Glu Tyr Tyr Asn Phe Asp Glu Trp Met 2160

ATC CAA TGG CAC AGG GAT ATC TGC AAG CCC TTT TCA ATT CCG TCG CCG GCA GCC AGG GCG 2160
Ile Gln Thr His Arg Asp Ile Cys Lys Pro Phe Ser Ile Pro Lys Pro Leu Glu Leu Leu 2220

CCG GGA TCT CCA GAA GCC CCG CCC ATC TTT CAG ATC TTT ATG GTC AAG TAC CTT TGC TCC 2220
Pro Gly Ser Pro Glu Ala Arg Pro Ile Phe Gln Ile Phe Met Val Lys Tyr Leu Cys Ser 2280

ATG TTG GTG GCG GCT ACT TCC AGC GTT TGG CTG TAT TCC AGC AAG ACG ATG GTC AGC TGG 2280
Met Leu Val Gly Val Thr Ser Ser Val Trp Leu Ser Ile Gly Phe Phe Ser Gly Leu Phe Leu 2340

CGG AAC TTC GTG GAG AGG TTG CAG GCG AAG GAG CCC CCG ACC CCG GCG CAG GCG TAC GTC 2340
Arg Asn Phe Val Glu Arg Leu Gln Gly Lys Glu Pro Arg Thr Arg Ala Gln Ala Tyr Val 2400

TAG TAT GAG ACG GGT CCG GCG GCG GCG GCC AAG TCC ACG CCC CTT ACT CCC GAT CCG GAG 2400
ter
GCG GCT AAC GAA AGT TAC TTA GAG TTT AGC ACA TAG ACG TTC CCT ATC CCG AGA AAA AAA 2460
AAA AAA

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FIG. 1 The sequence of the insert of the cDNA clone CVC22. The 2,466-bp sequence CVC22 is shown with the deduced translation product from the single long open reading frame. This cDNA clone was isolated from a library constructed by L. Kauvar¹⁸ from poly(A)⁺ RNA isolated from early pupae. The open reading frame has typical *Drosophila* codon usage, and the CAAA sequence preceding the ATG translation start sequence fits the *Drosophila* consensus sequence of C/AAAA/C¹⁹. There are termination codons in all three reading frames upstream from this start site. There is no typical poly(A) addition sequence (AATAAA) upstream of the 3' oligo(A) sequence, and the 3' untranslated region is relatively short, ~120 nucleotides. A more extensive cDNA clone analysis W. J. Park and P.N.A., unpublished results) has shown that CVC22 is missing over 1.3 kb of sequence of its 3' end. The analysis of additional cDNA clones from other libraries has not revealed any additional *fz* mRNA species in larvae or pupae. The putative transmembrane domains

are underlined and labelled. Sites of potential N-linked glycosylation are marked by an asterisk. The cDNA sequences were subcloned into the Bluescript vector (Stratagene), a set of processive deletions made, and single-stranded templates sequenced using the Sequenase reagents (US Biochemicals). The entire sequence was determined in both directions.

membrane domains *in vivo*, then the Fz protein would contain four extracellular and four intracellular domains. The largest of these would be the extracellular N-terminal domain. The sequences of three transmembrane domains (M4, M5 and M6, Fig. 1) are interesting because they contain a proline. Prolines are found in many transmembrane domains of membrane transport proteins¹⁰ and cell-surface receptors¹¹, whereas they are excluded from the transmembrane domains of other classes of membrane proteins, such as viral envelope proteins¹⁰. There is no striking sequence similarity between Fz and any of the sequences in the NBRF and translated GENBANK data bases¹².

The function of the *fz* locus is required for the transmission of polarity information. The predicted structure of the Fz protein

suggests it could do this either by transporting a polarity morphogen¹³ across cell membranes, or by mediating direct cell-cell interactions^{14,15}. The *fz* locus function is also required for cells to be able to respond to polarity information. The presence of seven putative transmembrane domains in the Fz protein is notable as seven transmembrane domains are found in the large family of G-protein-coupled membrane receptors^{11,16,17}. Although Fz does not have substantial sequence similarity to the products of any members of this gene family, it does seem to have topological similarity. It is therefore possible that Fz functions by a G protein to transduce polarity information to the cytoskeleton during hair and bristle development. □

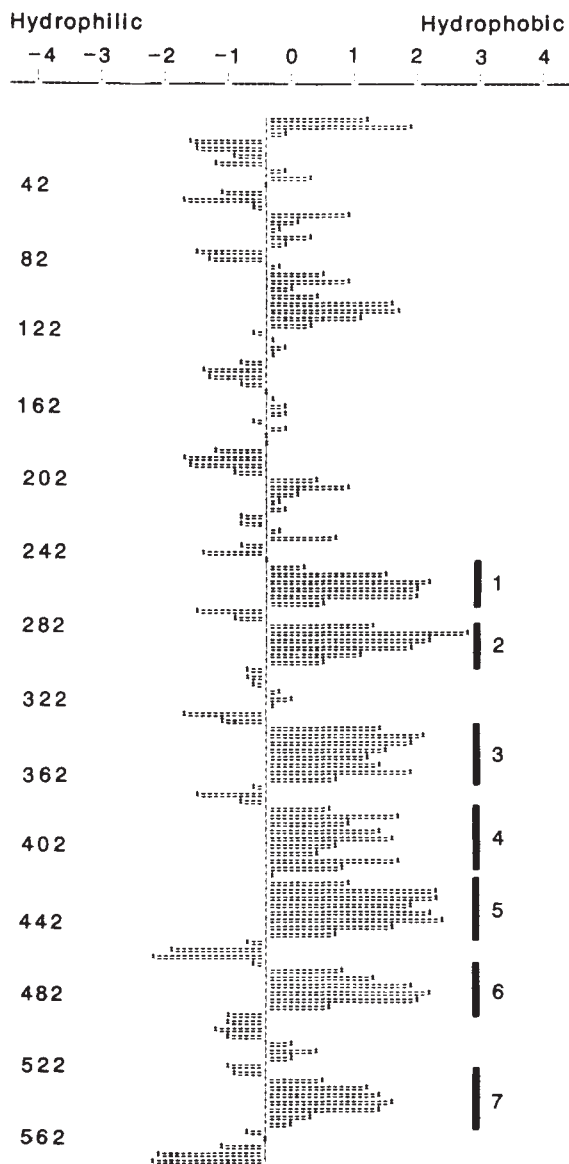


FIG. 2 Hydropathy plot of the Fz protein. Hydropathy of the deduced protein sequence was analysed as described in ref. 7, using windows of 9 and 19 residues, and by the GES approach⁸ with a window of 20 amino acids. The plot shown was generated by the approach of Kyte and Doolittle⁷ using a window of 9. The data is compressed four times, thus each point is an average for four residues. All analyses gave reasonably similar results except that the relatively hydrophobic region centred around amino acid 110 shown here came out much less hydrophobic when the GES approach was used (and somewhat less hydrophobic when the Kyte and Doolittle approach was used with a window of 19), so this region probably does not represent a transmembrane domain. The location of the seven proposed transmembrane domains is shown (1 to 7).

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- Gubb, D. & Garcia-Bellido, A. *J. Embryol. exp. Morph.* **68**, 37–57 (1982).
- Adler, P. N., Charlton, J. & Vinson, C. *Dev. Genet.* **8**, 99–119 (1987).
- Vinson, C. R. & Adler, P. N. *Nature* **329**, 549–551 (1987).
- Bingham, P. M., Levis, R. & Rubin, G. M. *Cell* **25**, 693–704 (1981).
- Searles, L. L., Jokest, R. S., Bingham, P. M., Voelker, R. A. & Greenleaf, A. L. *Cell* **31**, 585–592 (1982).
- Vinson, C. R. thesis Univ. Virginia (1987).
- Kyte, J. & Doolittle, R. F. *J. molec. Biol.* **157**, 105–132 (1982).
- Engelman, D. M., Steitz, T. A. & Goldman, A. A. *Rev. biophys. Chem.* **15**, 321–353 (1986).
- von Heijne, G. *J. molec. Biol.* **184**, 99 (1985).
- Brandl, C. J. & Deber, C. M. *Proc. natn. Acad. Sci. U.S.A.* **83**, 917–921 (1986).
- Dohlman, H. G., Caron, M. G. & Lefkowitz, R. J. *Biochemistry* **26**, 2657–2663 (1987).
- Pearson, W. R. & Lipman, D. J. *Proc. natn. Acad. Sci. U.S.A.* **85**, 2444–2448 (1988).
- Lawrence, P. A. *J. exp. Biol.* **49**, 607–620 (1966).
- Nardi, J. B. & Kafatos, F. V. *J. Embryol. exp. Morph.* **136**, 489–512 (1976).
- Tucker, J. B. *J. Embryol. exp. Morph.* **65**, 1–18 (1981).
- Gilman, A. G. A. *Rev. Biochem.* **56**, 615–649 (1987).
- Kobilka, B. K. *et al. Science* **240**, 1310–1316 (1988).
- Poole, S. J., Kauvar, B. D. & Kornberg, T. *Cell* **40**, 37–43 (1985).
- Cavener, D. R. *Nucleic. Acids Res.* **15**, 1353–1361 (1987).

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A *Pseudomonas* thrives in high concentrations of toluene

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TOLUENE, like many organic solvents, is highly biotoxic and kills most microorganisms at low concentrations (0.1% v/v). It is often used therefore to sterilize microbial cultures and lyse bacterial cells in the assay of bacterial enzymes^{1–3}. The physiological basis of such solvent toxicity, however, remains poorly characterized. Although some microorganisms, including *Pseudomonas*^{4–6}, *Achromobacter*⁴ and *Nocardia*⁷, can assimilate toluene, their tolerance for the solvent is less than 0.3% (v/v). We report here the discovery of a variant strain of *Pseudomonas putida* which is capable of growing in media culture containing more than 50% (v/v) toluene or high concentrations of cyclohexane, xylene, styrene and heptanol. By studying this unusually tolerant strain we show that the relative toxicities of different solvents are determined by their polarities.

We isolated the toluene-resistant bacterium from Japanese soil by selecting for microbial growth in a nutrient medium containing toluene. To isolate toluene-resistant microorganisms, a small amount of soil was suspended in sterile water and 0.2 ml of this suspension transferred to test tubes containing 5 ml of a nutrient medium comprising 0.1% glucose, 0.25% yeast extract (Difco Lab.) and 0.5% polypeptone (Difco Lab.), at pH 7.0. Toluene was then added to a final concentration of 30% (v/v), the test tubes were plugged with butyl-rubber, and the cultures incubated at 37°C for one week in a test-tube shaker. From the

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750 soil samples collected, one strain, IH-2000 (FERM BP-1751) which grew well in the toluene medium, was isolated from soil in Kyushu, southern Japan. Subsequent characterization of this bacterium showed that it is aerobic, motile, gram-negative and rod-shaped (0.8–1.0 by 2.0–4.0 μm), and has four polar flagella. The G and C content of its DNA is 62.5% and it reacts positively in catalase and oxidase tests, as well as being OF test-oxidative. From these and other morphological and biochemical characteristics of the strain, determined in accordance with standard methods⁸, the isolate IH-2000 was identified as a strain of *P. putida*, which differs from other strains only by its resistance to

toluene. Unlike *P. putida* strains which carry the TOL plasmid, strain IH-2000 is however, unable to utilize toluene as a nutrient.

By contrast with other strains including *P. putida* IFO 3738 and *P. putida* IAM 1506, which when tested for solvent tolerance failed to grow in a culture medium containing 30% toluene, strain IH-2000 grew in emulsions containing more than 50% toluene and was actively motile in these culture media. The doubling time for growth in a Luria-Bertani (LB) medium was 1.22 h and 0.77 h in the presence of 30% toluene and in the absence of the solvent, respectively (Fig. 1). After 24 h, the LB-toluene medium contained 2×10^9 cells per ml. Furthermore, single cells of this strain formed colonies on a solid nutrient medium, overlaid with toluene, which remained viable for several weeks. This indicates that solvent tolerance is a stable phenotypic property of strain IH-2000.

To investigate the solvent tolerance genetically, we isolated solvent-sensitive mutants using the replica-plating method in conjunction with penicillin selection after treatment with 1-methyl-3-nitro-1-nitrosoguanidine (NTG). First, we made Leu and Trp auxotrophs from the IH-2000 strain by two NTG treatments. We then isolated three toluene-sensitive mutants from 3,000 colonies of mutant cells. Remarkably, one of these (IH-2124T) was sensitive to toluene but resistant to xylene and cyclohexane. We subsequently isolated one xylene-sensitive mutant (IH-2124TX) from 2,000 colonies of mutant cells derived from the toluene-sensitive strain. This strain was consequently sensitive to both toluene and xylene, but resistant to cyclohexane. From the IH-2124TX strain, a cyclohexane-sensitive mutant (IH-2124TxC) which remained hexane-resistant was isolated. Each of these mutants retained the Leu and Trp auxotrophic markers. The toluene-sensitive mutant, for which the frequency of back mutation was less than 10^{-9} , was very stable. The xylene-sensitive and cyclohexane-sensitive mutants exhibited frequencies of back mutation of 10^{-7} to 10^{-8} respectively. In addition to toluene, these strains tolerated other toxic solvents including aliphatic hydrocarbons, alicyclic hydrocar-

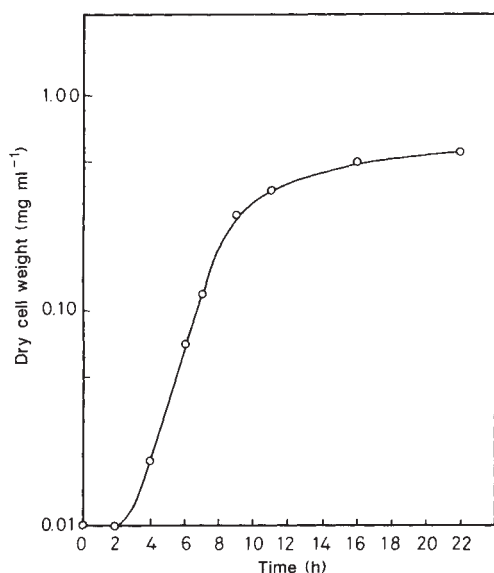


FIG. 1 Growth curve of strain IH-2000 in the LB medium containing 30% toluene.

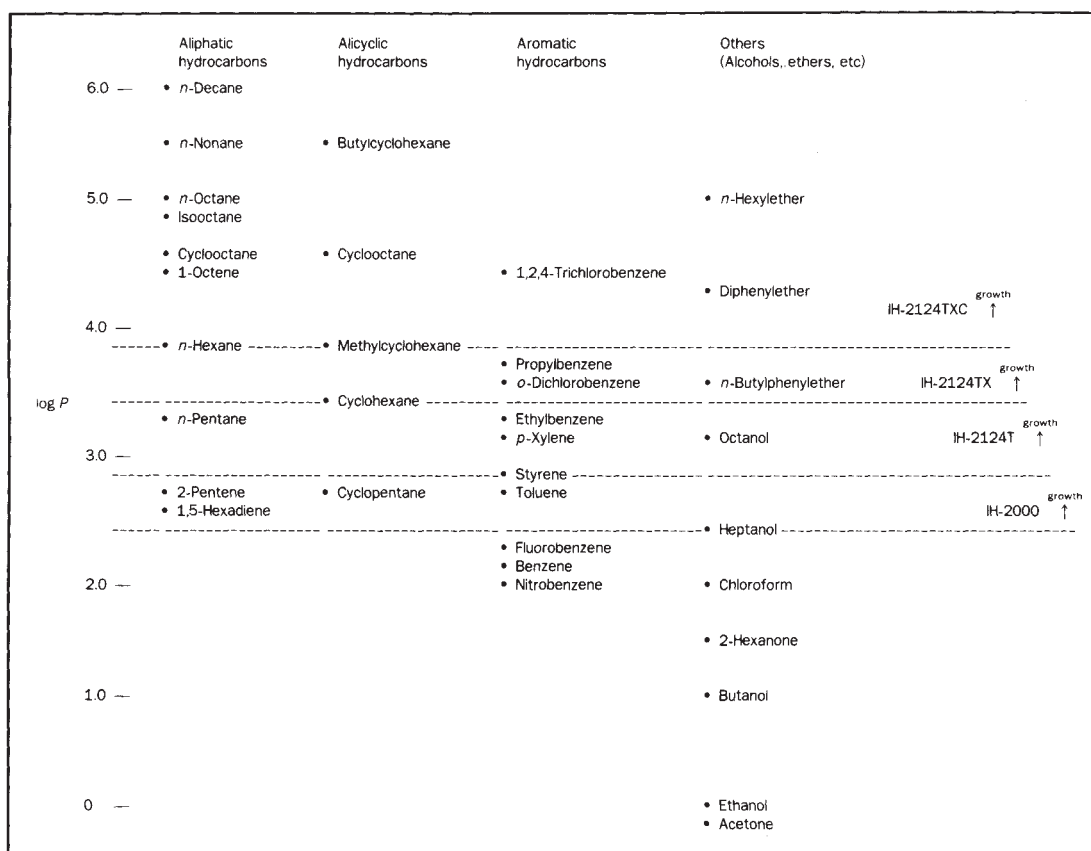


FIG. 2 Correlation between log *P* values and growth limits of the strain IH-2000 and its mutants in media-containing solvents. Log *P* values were calculated using the CLOG program, 3.33 (Pomona College, USA).

TABLE 1 Solvent tolerance of bacterial strains and mutants

	log P	IH-2000	IH-2124T	IH-2124TX	IH-2124TXC	<i>E. coli</i> IFO 3806	<i>P. putida</i> IFO 3738	<i>P. fluorescens</i> IFO 3507	<i>Achromobacter</i> <i>delicatulus</i> IAM 1433	<i>Agrobacterium</i> <i>tumefaciens</i> IFO 3058	<i>Alcaligenes faecalis</i> JCM 1474	<i>Aeromonas hydrophila</i> JCM 1027	<i>B. subtilis</i> AHU 1219	<i>Corynebacterium</i> <i>glutamicum</i> JCM 1318	<i>S. uvarum</i> ATCC 26602	No. tolerant strains
Dodecane	7.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	14
Decane	6.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	12
Nonane	5.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	12
n-Hexylether	5.1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	12
Octane	4.9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	12
Isooctane	4.8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	11
Cyclooctane	4.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	10
Diphenylether	4.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	8
n-Hexane	3.9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	8
Propylbenzene	3.8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	6
o-Dichlorobenzene	3.6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	5
Cyclohexane	3.4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	5
Ethylbenzene	3.3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	3
p-Xylene	3.1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	3
Styrene	2.9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	2
Toluene	2.8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
Heptanol	2.4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
Benzene	2.1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0
Limiting log P value for growth		2.4	2.9	3.4	3.9	3.8	3.1	3.4	3.9	4.8	4.5	4.5	4.9	7.0	7.0	

+, growth —, no growth

bons, aromatic hydrocarbons, alcohols and ethers. The solvent tolerance properties of the IH-2000 and its mutants are summarized in Table 1.

Figure 2 shows the correlation between the growth of strain IH-2000 and the polarity of the solvent. The parameter log *P*, where *P* is the partition coefficient of a given solvent in an equimolar mixture of octanol and water, is used as a quantitative index of solvent polarity. It is evident that although the strain grows in solvents whose log *P* values are greater than or equal to 2.4, such as heptanol (log *P* = 2.4), toluene (log *P* = 2.8) and n-octane (log *P* = 4.9), it does not grow in fluorobenzene, benzene and butanol whose log *P* values are 2.3, 2.1 and 0.8, respectively. It seems therefore that a critical point is reached between log *P* values of 2.4 and 2.3, at which the solvent is polar enough to prevent growth. The concept of such a tolerance limit can be extended to predict the growth of other microbial species in solvent-saturated environments (see Table 1).

These observations indicate that the nature of the interaction between the solvent and the cell surface that limits growth is at least partly determined by solvent polarity. Moreover, the unusually high tolerance of strain IH-2000 for toluene and other solvents probably reflects the presence of cell surface components that are unique to the strain. With a view to characterizing these components and their genetic basis, we have already isolated, from the chromosomal DNA of strain IH-2000, a 10-kilobase fragment (data not shown) which contains genes for solvent tolerance. □

Phase variation in *Bordetella pertussis* by frameshift mutation in a gene for a novel two-component system

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BORDETELLA pertussis, the aetiological agent of whooping cough, coordinately regulates the expression of many virulence-associated determinants, including filamentous haemagglutinin, pertussis toxin, adenyl cyclase toxin, dermonecrotic toxin and haemolysin. The coordinate regulation is apparent in the repression of synthesis of these determinants in response to environmental stimuli^{1,2}; a phenomenon known as antigenic or phenotypic modulation. *B. pertussis* also varies between metastable genetic states, or phases. There is a virulent phase in which virulence-associated determinants are synthesized, and an avirulent phase in which they are not³. Previous studies^{4,5} have shown that a genetic locus, *vir*, is required for expression from many virulence-associated loci, and that replacing the cloned *vir* locus in *trans* can restore the virulent phase phenotype to spontaneously occurring avirulent phase strains. Here, we show that phase variation in one series of strains is due to a frameshift mutation within an open reading frame that is predicted to code for a Vir protein product. The deduced protein sequence is similar to both components of the 'two-component' regulatory systems which control gene expression in response to environmental stimuli in a range of bacterial species.

Tohama I is a virulent phase *B. pertussis* strain isolated from a pertussis patient in Japan⁶. The ancestry of all *B. pertussis* strains used in this study can be traced to this strain, and their

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1. Herzenberg, L. A. *Biochim. biophys. Acta*, **31**, 525-539 (1959).
2. Dobrogosz, W. J. & DeMoss, R. D. *J. Bact.* **85**, 1350-1365 (1963).
3. Leventhal, C., Singer, E. R. & Fetherhor, K. *Proc. natn. Acad. Sci. U.S.A.* **48**, 1230-1238 (1962).
4. Worsey, M. J. & Williams, P. A. *J. Bact.* **124**, 7-13 (1975).
5. Kitagawa, M. *J. Biochem., Tokyo* **43**, 553-563 (1956).
6. Claus, D. & Walker, N. *J. gen. Microbiol.* **36**, 107-122 (1964).
7. Raymond, R. L., Jamison, V. W. & Hudson, J. O. *Appl. Microbiol.* **15**, 857-865 (1967).
8. *Bergey Manual of Systematic Bacteriology* (Williams & Wilkins, 1984).
9. Corwin, H. & Anderson, M. *J. org. Chem.* **32**, 2583-2586 (1967).
10. Rekker, R. F. & de Kort, H. M. *Eur. J. med. Chem. chim. Therapeut.* **14**, 479-488 (1979).
11. Harnisch, M., Mockel, H. J. & Schulze, G. *J. Chromatogr.* **282**, 315-332 (1983).

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relationship is shown in Fig. 1. We have shown previously that a 14.7-kilobase (kb) *Bam*HI fragment from BP338 containing the *vir* locus can restore the virulent phase phenotype to BP369-3 when returned in *trans*⁵. We repeated this experiment for the other strains shown in Fig. 1 by cloning the homologous *Bam*HI fragment from BP326, BP368-3, BP369-3, BP370-1 and BP371-1 into the broad host-range cloning vector pRK290 (ref. 7) and transferring the resultant plasmids by conjugation to the avirulent phase strains BP326, BP369-3 and BP371-1. When the *vir* locus transferred had been cloned from one of the virulent phase strains BP338, BP368-3 or BP370-1, the resulting exconjugant colonies had a typically *Vir*⁺ phenotype, being compact hemispherical (domed), and with a clear zone of haemolysis on Bordet-Gengou agar. When the *vir* locus was from BP326, BP369-3 or BP371-1, a *Vir*⁻ phenotype was obtained (flat and nonhaemolytic).

We examined the virulent phase (*vir*⁺ form) and the avirulent phase (*vir*⁻ form) of the cloned *vir* locus to see if there was a physical difference, such as an inversion or deletion that could explain the genetic differences. None were apparent after restriction enzyme analysis with *Eco*RI, *Sal*I or *Pst*I, or electron microscopic heteroduplex analysis (data not shown). We therefore designed an experiment to map the location of this difference by genetic means.

We constructed a set of deletions of the *vir* locus of BP338 *in vitro* (Fig. 2), and tested them for their ability to restore the *Vir*⁺ phenotype in two different ways. For the test in *trans*, each derivative was cloned into pRK290 and returned by conjugation to a virulent phase *B. pertussis* strains. None of the four deletions tested contained an intact *vir* locus, as they were all negative in *trans* (data not shown). To test for restoration of the *Vir*⁺ phenotype by recombination, the same deletion fragments were cloned into pSS853, a vector that confers kanamycin resistance. This vector can be transferred by conjugation into *B. pertussis* but cannot replicate there autonomously. Thus, exconjugants can be selected in which the plasmid has been integrated into the *B. pertussis* chromosome by selection for kanamycin resistance. This integration will probably occur by homologous recombination within an area of homology; in the case of the *vir* deletion derivatives, within the cloned *vir* DNA. If the deletion derivative being tested contains the site of the difference between the *vir*⁺ and *vir*⁻ forms then some of the exconjugants arising from a mating will be *Vir*⁺ due to crossover between this site and the end-point of the deletion. If the deletion derivative does not contain the site of this difference, only *Vir*⁻ colonies will be obtained. As shown in Fig. 2, two of the deletions could restore the virulent phase phenotype by recombination. These data indicate that the difference between the *vir*⁺ and *vir*⁻ forms of the *vir* locus lies within the deletion interval defined by deletions 856 and 858, within the *Sal*I fragment D. The same results were obtained regardless of which avirulent phase strain was the recipient; hence, the *vir*⁻ lesion is within this deletion

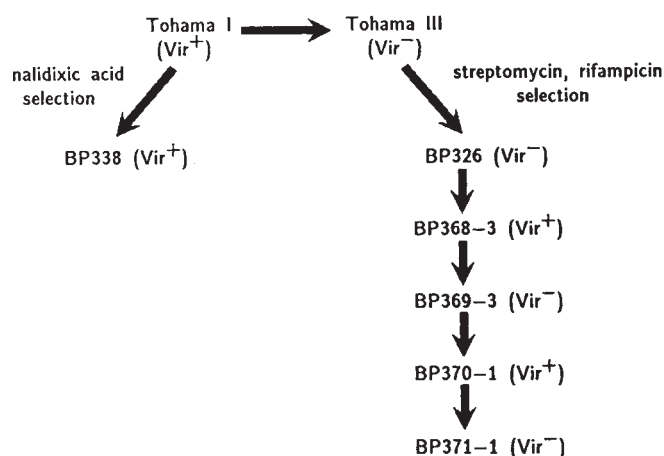


FIG. 1 Pedigree of *B. pertussis* strains used in this study. Tohamia I was isolated from a patient in Japan⁶. BP338 is a spontaneous nalidixic acid-resistant derivative of Tohamia I¹². Tohamia III was isolated as a spontaneous variant of Tohamia I after passage *in vitro* (Y. Sato, personal communication). BP326 is a spontaneous rifampicin and streptomycin-resistant derivative of Tohamia III¹². BP368-3 and BP370-1 were isolated as spontaneously occurring haemolytic variants of BP326 and BP369-3, respectively^{4,8}. BP369-3 and BP371-1 were isolated from BP368-3 and BP370-1, respectively, by erythromycin selection for avirulent phase derivatives^{4,5}.

interval in BP326, BP369-3 and BP371-1. To establish further that the difference between the *vir*⁺ and *vir*⁻ forms was contained within *Sal*I restriction fragment D, this fragment was used in a double crossover recombination experiment to rescue the *Vir*⁻ phenotype. It was cloned into pRTP1, a vector designed for the replacement of chromosomal sequences with cloned and/or modified ones⁸ (Fig. 2). The resulting plasmid was modified further by the addition of a gene encoding kanamycin resistance to improve the selection for exconjugants. This plasmid, pSS1064, was transferred by conjugation to BP326, BP369-3 and BP371-1. The resulting kanamycin-resistant *B. pertussis* derivatives were of the avirulent phase, as judged by colony morphology and we assumed that the plasmid had integrated via a single crossover within the cloned *Sal*I fragment. In a sampling of these *vir*⁻ exconjugants, we selected for a second crossover event by selecting for loss of the integrated plasmid by virtue of the streptomycin sensitivity phenotype it conferred. The colonies arising after streaking on streptomycin-containing media were both *Vir*⁺ and *Vir*⁻, as expected. The two types of colonies were obtained in approximately equal numbers. The frequency at which *vir*⁺ derivatives arise by this method is at least 1,000-fold higher than the frequency at which they arise spontaneously. This experiment confirmed that the site of the functional difference in the two forms of the *vir* locus is within the *Sal*I fragment D.

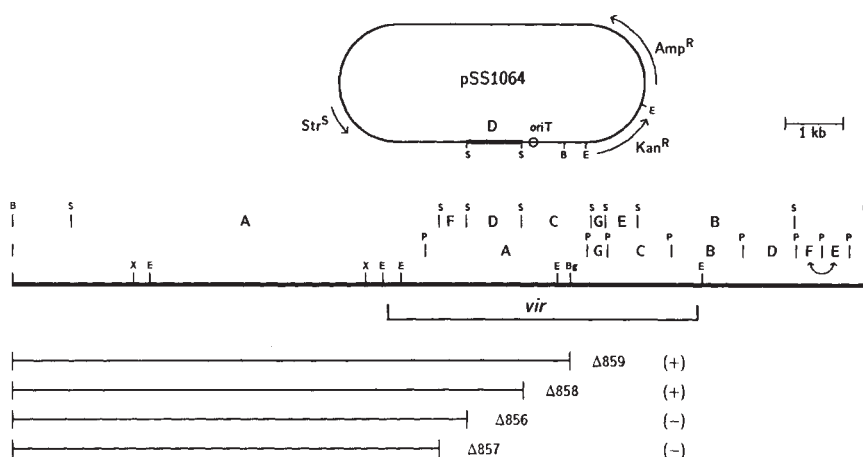


FIG. 2 Restriction map of the *vir* locus of *B. pertussis*. Restriction enzyme abbreviations: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sal*I; X, *Xho*I. The bracket below the map shows the extent of the *vir* locus determined by insertion and deletion mapping in *B. pertussis* (manuscript in preparation). DNA sequences remaining in the deletion derivatives are shown below the map: '+' indicates that a given deletion was capable of restoring, by recombination, the virulent phase phenotype (domed and haemolytic) when cloned into pSS853 and transferred from *Escherichia coli* strain SM10 (ref. 13) to BP326 by conjugation. pSS853 is a derivative of pBR322 containing *oriT* from RK2, and a kanamycin-resistance gene. Shown above the map is the structure of pSS1064; derived by cloning the *Sal*I fragment D of *vir* from BP338 into the vector pRTP1 (ref. 8) and subsequent addition of a kanamycin-resistance cassette.

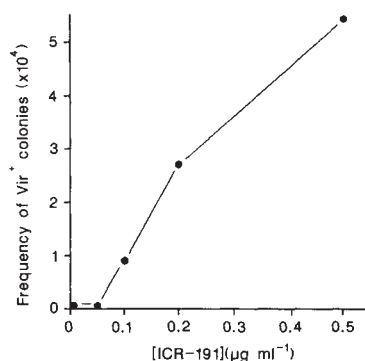


FIG. 4 Reversion of the avirulent phase phenotype of BP326 by treatment with the frameshift mutagen ICR-191. The procedure was essentially as previously described²⁴. A culture of BP326 grown in Stainer-Scholte broth was diluted by 10^{-4} . Aliquots (0.1 ml) were added to 3 ml of the same medium containing different concentrations of ICR-191 and incubated in the dark at 35 °C, with shaking, for 5–7 days. Cultures showing visible turbidity were diluted and plated on Bordet-Gengou agar to obtain 10^3 – 10^4 colonies per plate. After growth for 5 days, plates were examined for haemolytic colonies.

Similar results were obtained for BP369-3 and BP371-1. These data support the hypothesis that phase variation in this series of strains occurs via a frameshift mutation in a *vir* gene.

An important question arising from our results is whether this frameshift mutation represents a true 'programmed' genetic event reflecting an evolved site-specific mechanism. The exact nucleotide sequence requirements at the site of the mutation are not yet known. It is worth noting that in *B. pertussis*, an organism with a relatively high (G+C) content, a run of six G or six C residues is, as expected, not uncommon. The sequence around the site of the frameshift mutation is striking however, in its abundance of GC pairs and in its near symmetry. It will be interesting to test whether the groups of C residues outside the core six C residues affect the frequency of phase variation. This frequency varies with the strain used and is relatively high in the strains which have descended from Tohama III (about 10^{-3} for the *vir*⁺ to *vir*⁻ transition⁴). In contrast, the frequency at which BP338 undergoes this transition is about 10^{-6} . In an attempt to determine the basis for this difference, we are examining the reversion of other frameshift mutations in these two backgrounds to determine whether this is a general effect on frameshift mutation/reversion. The relevance of our results, derived from work on laboratory strains, to bacteria in their natural environment is an important issue. It is unclear what the role of phase variation is in the natural biology of this highly adapted pathogen. This phenomenon has been observed after passage of *B. pertussis* isolates *in vitro* in most cases. The isolation of *Vir*⁻, or phase III, organisms from patients late in infection has been reported however⁶. We are currently examining *vir*⁺/*vir*⁻ pairs derived from other isolates to determine if the frameshifting we have described represents a common mechanism for phase variation in *Bordetella* species. □

18. Comeau, D. E., Ikenaka, K., Tsung, K. & Inouye, M. *J. Bact.* **164**, 578–584 (1985).
19. Mutoh, N. & Simon, M. I. *J. Bact.* **165**, 161–166 (1986).
20. Ferrari, F. A. et al. *Proc. natn. Acad. Sci. U.S.A.* **82**, 2647–2651 (1985).
21. Trach, K. A., Chapman, J. W., Piggot, P. J. & Hoch, J. A. *Proc. natn. Acad. Sci. U.S.A.* **82**, 7260–7264 (1985).
22. Drury, L. S. & Buxton, R. S. *J. biol. Chem.* **260**, 4236–4242 (1985).
23. Lipman, D. J. & Pearson, W. R. *Science* **227**, 1434–1441 (1985).
24. Miller, J. *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, New York (1972)).
25. Kofoid, E. C. & Parkinson, J. S. *Proc. natn. Acad. Sci. U.S.A.* **85**, 4981–4985 (1988).

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A new strategy for the generation of catalytic antibodies

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THE high binding affinity and specificity of antibodies for a wide range of ligands has recently been exploited in the generation of catalysts for acyl-transfer reactions^{1–7}, carbon–carbon bond forming^{8,9} and carbon–carbon bond cleaving reactions¹⁰. In addition, a number of strategies are emerging for the generation of catalytic antibodies including transition state stabilization^{1–6}, catalysis by approximation^{7,8}, and the introduction of catalytic groups or cofactors into antibody combining sites^{10–13}. An important goal in the design of catalytic antibodies is the development of general rules relating hapten structure to the corresponding catalytic groups in the antibody combining site. We report here that electrostatic interactions between a hapten and the complementary antibody^{14,15} can be exploited to generate catalytic amino-acid side chains in an antibody-combining site. The antibody-catalysed reaction, a β -elimination reaction, exhibits saturation kinetics, substrate specificity, competitive inhibition by hapten, and specific inactivation by a reagent that modifies carboxylate residues.

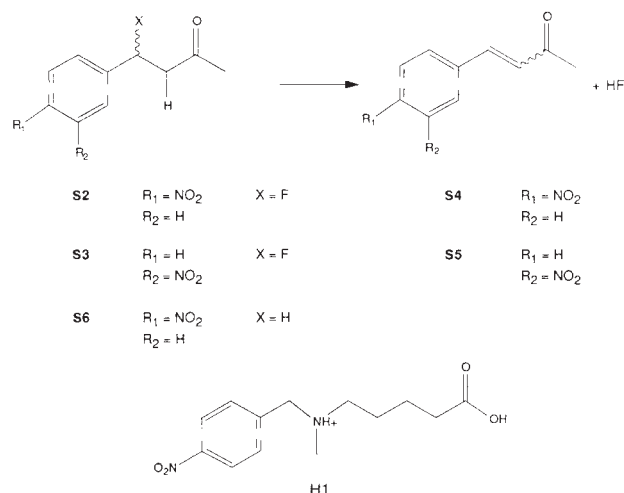
To expand the scope of antibody catalysis we targeted reactions involving proton abstraction from a carbon centre. This class of reactions includes elimination and isomerization reactions and aldol and Claisen condensations^{16–18}. Proton abstraction in enzymes is often performed by carboxylate side chains of glutamate or aspartate amino acids. These residues typically display higher than normal pKa values (6.5–8.2) in the hydrophobic active sites of enzymes¹⁹. Aspartate and glutamate residues have been generated in the combining sites of antibodies by taking advantage of charge complementarity between haptens and the corresponding antibodies^{14,15}. Consequently, we reasoned that an antibody-combining site could be generated that contains a carboxylate group within bonding distance of an abstractable substrate proton by using the appropriate positively charged hapten. Importantly, the position of a charged group such as alkyl ammonium ion in the hapten should reflect that of the target C–H group in the substrate.

To test these notions, monoclonal antibodies generated to hapten H1 (Fig. 1) were assayed for their ability to catalyse H–F elimination from the fluorinated substrate S2 (Fig. 1). The hapten contains a positively charged ammonium ion replacing the α -CH₂ group of the substrate (the tertiary nitrogen obviates competing lactam formation during the coupling of H1 to pro-

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1. Idigbe, O. R., Parton, R. & Wardlaw, A. C. *J. med. Microbiol.* **14**, 409–418 (1981).
2. Lacey, B. W. *J. Hyg.* **58**, 57–93 (1960).
3. Leslie, P. H. & Gardner, A. D. *J. Hyg.* **31**, 423–434 (1931).
4. Weiss, A. A. & Falkow, S. *Infect. Immunity* **43**, 263–269 (1984).
5. Stibitz, S., Weiss, A. A. & Falkow, S. *J. Bact.* **170**, 2904–2913 (1988).
6. Kasuga, T., Nakase, Y., Ukishima, K. & Takatsu, K. *Kitasato Arch. Exp. Med.* **27**, 57–62 (1954).
7. Ditta, G., Stanfield, S., Corbin, D. & Helinski, D. R. *Proc. natn. Acad. Sci. U.S.A.* **77**, 7347–7351 (1980).
8. Stibitz, S., Black, W. & Falkow, S. *Gene* **50**, 133–140 (1986).
9. Roth, J. R. *A. Rev. Genet.* **8**, 319–346 (1974).
10. Ronson, C. W., Nixon, T. B. & Ausubel, F. M. *Cell* **49**, 579–581 (1987).
11. Parkinson, J. S. *Cell* **53**, 1–2 (1988).
12. Weiss, A. A., Hewlett, E. H., Myers, G. A. & Falkow, S. *Infect. Immunity* **42**, 33–41 (1983).
13. Simon, R., Priefer, U. & Puhler, A. *Biotechnology* **1**, 784–791 (1983).
14. David, M. et al. *Cell* **54**, 671–683 (1988).
15. Ronson, C. W., Astwood, P. M., Nixon, B. T. & Ausubel, F. M. *Nucleic Acids Res.* **15**, 7921–7934 (1987).
16. Makino, K., Shinagawa, H., Amemura, M. & Nakata, A. *J. molec. Biol.* **192**, 549–556 (1986).
17. Amemura, M., Makino, K., Shinagawa, H. & Nakata, A. *J. Bact.* **168**, 294–302 (1986).

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tein carriers for immunization). The fact that the hapten and substrate share a common recognition element, the *p*-nitrophenyl group, ensured that the antibody-binding affinity for the substrate would be reasonable. Moreover, replacement of hapten by substrate in the antibody-combining site should lead to an increase in the basicity of the catalytic carboxylate since a stabilizing salt bridge interaction is lost.

Antibodies were generated against hapten H1, which at physiological pH exists as a positively charged alkyl ammonium ion. The hapten was conjugated to the carrier proteins bovine serum albumin (BSA) and keyhole limpet haemocyanin (KLH) using the activated *N*-hydroxysuccinimide ester of H1 (ref. 20). Six monoclonal antibodies specific for H1 were produced *in vivo* and isolated by affinity chromatography on protein A-coupled Sepharose 4B as described previously³. Antibody purity was determined by 10–15% SDS polyacrylamide gel electrophoresis with Coomassie blue staining²¹. Of these six IgGs isolated, four accelerated the β -elimination reaction of S2 to S4 (Fig. 1) and were completely inhibitable by the addition of free

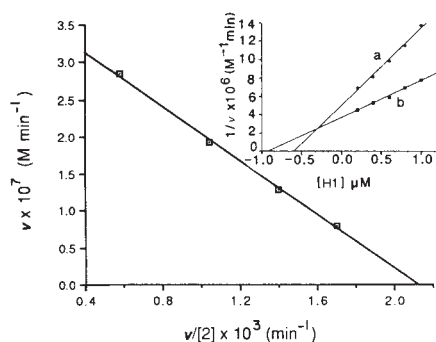


FIG. 2 Eadie-Hofstee plot for the β -elimination conversion of S2 to S4, catalysed by 43D4-3D3. Velocities were determined spectrophotometrically by measuring the initial absorbance increase at 330 nm: S2, $\lambda_{\text{max}} = 282$ nm (3.99); S4 $\lambda_{\text{max}} = 312$ nm (4.43); $\Delta E(\text{S4-S2})$ (330 nm) = $16,820 \text{ cm}^{-1} \text{ M}^{-1}$. The concentration of 43D4-3D3 was $2.00 \mu\text{M}$ as determined by absorbance at 280 nm using $E(1 \text{ cm}, 0.1\%) = 1.37$ and $M_r = 150,000$ for IgG. 43D4-3D3 was preincubated at 37°C in 10 mM bis [2-hydroxyethyl]imino-tris-[hydroxymethyl]-methane (bis-Tris), 100 mM NaCl, pH 6.0. The reaction was initiated by adding $10 \mu\text{l}$ of a stock solution of S2 in CH_3CN to give a final CH_3CN concentration of 2%. The uncatalysed rate was measured under the same conditions, affording a $k_{\text{uncat}} = 1.30 \times 10^{-4} \text{ min}^{-1}$. Inset: Dixon plot²⁶ of inhibition of 43D4-3D3 by H1. Antibody concentration, $2.01 \mu\text{M}$. Data was obtained at two concentrations of S2: a, $208 \mu\text{M}$; b, $514 \mu\text{M}$. Buffer conditions as above.

FIG. 1 Monoclonal antibodies elicited against the (KLH) conjugate of H1 catalyse the conversion of S2 or S4. Synthesis of H1 was accomplished by treatment of *p*-nitrobenzaldehyde and β -aminovaleic acid with 0.7 equivalents (eq.) of sodium cyanoborohydride (NaCNBH_3) in methanol at 25°C to afford *N*(*p*-nitrobenzyl)- β -aminovaleic acid which was purified by recrystallization from $\text{H}_2\text{O}/\text{acetone}$. This compound was then treated with 37% aqueous formaldehyde and 2 eq. NaCNBH_3 in H_2O at 25°C to afford H1 which was purified by silica-gel chromatography. Compound H1 was coupled through amide linkages to KLH by treatment with the *N*-hydroxysuccinimide ester of H1 to afford the KLH conjugate with an epitope density of 18 per carrier monomer as determined by the method of Habeeb²⁴; protein concentration was determined by the method of Lowry²⁵. Synthesis of S2 was carried out by treatment of *p*-nitrobenzaldehyde with a catalytic amount of 1% aqueous NaOH in acetone at 0°C to give the intermediate (*R,S*) 4-hydroxy-4-*p*-nitrophenylbutan-2-one which after purification by silica-gel chromatography, treatment with diethylaminosulphur trifluoride in CH_2Cl_2 at -78°C and aqueous extraction afforded racemic S2. Treatment of the intermediate alcohol with 1.1 eq. of H_2SO_4 (1N) in acetone at 25°C and purification by silica gel chromatography afforded the *trans* isomer of S4. Compounds S3 and S5 were synthesized analogously to S2 and S4 starting with 3-nitrobenzaldehyde. Substrate analogue S6 was obtained by nitration of benzylacetone with $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ in neat trifluoroacetic anhydride at 39°C and purified by silica gel chromatography. All compounds were characterized by NMR, mass spectrometry, infrared and ultraviolet spectroscopy and elemental analysis.

hapten H1. Such hapten-inhibitable rate acceleration suggests that the catalysis is due to binding of the substrate in the antibody-combining site (non-specific antibodies did not catalyse the elimination reaction). The fact that 66% of these antibodies were catalytic demonstrates the generality of this strategy for producing catalytic antibodies. One of the four antibodies (43D4-3D3) was characterized further.

The reaction catalysed by 43D4-3D3 obeys classical Michaelis-Menten kinetics: a k_{cat} of 0.193 min^{-1} and a K_M of $182 \mu\text{M}$ for substrate S2 were measured at pH 6.0 (Fig. 2). Both the *cis* and *trans* isomers of enone S4 are formed in the antibody-catalysed conversion of racemic S2, as determined by high pressure liquid chromatography (HPLC) analysis. The catalysed reaction is also competitively inhibited by hapten H1 ($K_i = 290 \text{ nM}$) (Fig. 2 inset) demonstrating that the catalytic activity is associated with binding in the antibody-combining site. An unreactive substrate analogue *p*-nitrobenzylacetone (S6) is also a competitive inhibitor of the catalysed reaction ($K_i = 280 \mu\text{M}$). As expected, the antibody-catalysed reaction is substrate-specific, in accordance with the characteristic specificity of antibodies for their ligands¹⁵. The K_M and k_{cat} values for 4-fluoro-4-*m*-nitrophenylbutan-2-one (S3) are $571 \mu\text{M}$ and 0.079 min^{-1} , respectively ($k_{\text{cat}}/K_M = 1.38 \times 10^2 \text{ M}^{-1} \text{ min}^{-1}$) at pH 6.50, compared with a k_{cat} of 0.304 min^{-1} and K_M of $214 \mu\text{M}$ ($k_{\text{cat}}/K_M = 1.42 \times 10^3 \text{ M}^{-1} \text{ min}^{-1}$) for S2 at this pH.

The identity of the catalytic amino-acid side chain in the antibody combining site was probed by two methods. The pH dependence of k_{cat} shows the classical profile for catalysis attributable to a single titratable group (Fig. 3). The pK_a for the active site residue was determined to be 6.2. This value is very close to the pK_a for the active-site Glu 135 in carboxypeptidase A ($\text{pK}_a = 6.5$), which is known to be responsible for the catalysis of a similar β -elimination reaction (not the physiologically relevant reaction for this enzyme)²². The maximum k_{cat} value obtainable by the antibody results when the residue responsible

TABLE 1 Effect of DAA treatment of 43D4-3D3 on catalysis of S2 to S4

Sample	v ($\text{M}^{-1} \text{ min}^{-1}$)*	% activity remaining
Untreated	3.41×10^{-7}	100
DAA treated†	7.97×10^{-8}	23.4
DAA treated† + 2 mM S6	2.79×10^{-7}	82.0

* $1.5 \mu\text{M}$ 43D4-3D3, $540 \mu\text{M}$ S2, pH 6.0, 10 mM Bis-Tris, 100 mM NaCl.

† Following the procedure described in ref. 14.

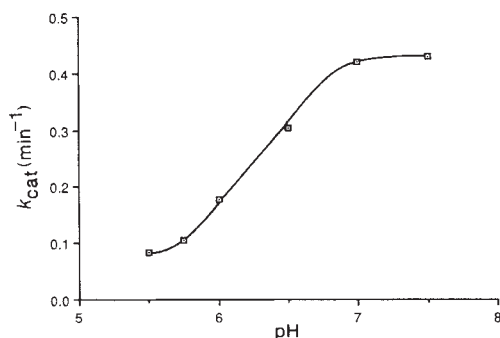


FIG. 3 The k_{cat} versus pH profile for conversion of S2 to S4 catalysed by 43D3-3D3. At each pH the k_{cat} value was obtained from a Lineweaver-Burke plot. The buffer used was 10 mM bis-Tris, in the presence of 100 mM NaCl.

for base catalysis is fully deprotonated. The value of $k_{cat}(\max)$, determined from the pH profile by plotting k_{cat} versus $k_{cat}[\text{H}^+]$, is 0.458 min^{-1} (ref. 23).

Chemical modification confirmed that an aspartate or glutamate residue is involved in the elimination reaction. Specific chemical modification of carboxylate residues was effected by treatment of 43D4-3D3 with diazoacetamide (DAA). DAA has been shown to react almost exclusively with carboxylate groups to form glycolamide ester linkages¹⁴. The antibody 43D4-3D3 retains only 23% of its catalytic activity after treatment with DAA. However, in the presence of competitive inhibitor S6 (2.0 mM), under otherwise identical conditions, 82% of its catalytic activity is retained. These results indicate that a carboxylate group could be located in or very near the binding site of 43D4-3D3.

The ability to generate antibody binding sites with specific catalytic groups or microenvironments provides a method for dissecting the contribution of various factors involved in enzymatic catalysis, such as transition-state stabilization, proximity effects, general acid-base, nucleophilic catalysis and ground state strain. Hapten H1 bears very little resemblance to the transition state for the conversion of S2 to S4; in fact it has the opposite charge of an E2 elimination transition state. This observation, together with the pH profile (Fig. 3), suggests that the observed catalysis is largely due to the presence of a catalytic base. The rate constant for acetate-catalysed conversion of S2 to S4, k_{OAc^-} , is $1.13 \times 10^{-2} \text{ M}^{-1} \text{ min}^{-1}$ at 37 °C in 100 mM NaCl. The k_{cat}/K_M value of the antibody-catalysed reaction at pH 6 is $9.95 \times 10^2 \text{ M}^{-1} \text{ min}^{-1}$. The rate enhancement obtained by introduction of a carboxylate residue in a hydrophobic binding site compared with acetate free in solution is therefore 8.80×10^4 at the pH. This value is similar to that of 6×10^4 reported for an esterolytic antibody that contains an active site nucleophilic thiol¹². The large rate enhancement due solely to base catalysis by a carboxylate residue suggests that an antibody which combines transition state stabilization and base catalysis might attain rate accelerations similar to those of enzymes. Nuclear magnetic resonance experiments and resolution of the *R*, *S* stereoisomers of S2 are currently in progress to determine the stereochemistry of proton abstraction (pro*R*, pro*S*) and elimination (*syn*-coplanar, *anti*-coplanar).

This work represents the extension of antibody catalysis to a new class of reactions. Moreover, it should be possible to exploit hapten complementarity to generate antibodies which contain other catalytic groups and catalyse a variety of reactions of interest, including aldol and Claisen condensations, as well as amide and sugar hydrolyses.

- Napper, A. D., Benkovic, S. J., Tramantano, A. & Lerner, R. A. *Science* **237**, 1041–1043 (1987).
- Janda, K. D., Schloeder, D., Benkovic, S. J. & Lerner, R. A. *Science* **241**, 1188–1191 (1988).
- Schultz, P. G. *Science* **240**, 426–433 (1988).
- Benkovic, S. J., Napper, A. D. & Lerner, R. A. *Proc. natn. Acad. Sci. U.S.A.* **85**, 5355–5358 (1988).
- Jackson, D. Y. *et al. J. Am. chem. Soc.* **110**, 4841–4842 (1988).
- Hilvert, D. & Nared, K. D. *J. Am. chem. Soc.* **110**, 5593–5594 (1988).
- Cochran, A. G., Sugawara, R. & Schultz, P. G. *J. Am. chem. Soc.* **110**, 7888–7890 (1988).
- Shokat, K. M., Leumann, C. J., Sugawara, R. & Schultz, P. G. *Angew. Chem. Intl. Edn Engl.* **27**, 1172–1174 (1988).
- Pollack, S., Nakayama, G. & Schultz, P. G. *Science* **242**, 1038–1040 (1988).
- Pollack, S. & Schultz, P. G. *J. Am. chem. Soc.* (in the press).
- Grossberg, A. L. & Pressman, D. *J. Am. Chem. Soc.* **82**, 5478–5482 (1960).
- Pressman, D. & Grossberg, A. L. in *The Structural Basis of Antibody Specificity* 166–174 (Benjamin, New York, 1968).
- Schrag, K. J., O'Connell, E. L. & Rose, I. A. *J. biol. Chem.* **248**, 2214–2218 (1973).
- Banner, D. W. *et al. Nature* **255**, 609–614 (1975).
- Miziorko, H. M. & Lane, M. D. *J. biol. Chem.* **252**, 1414–1420 (1977).
- Parsons, S. M. & Raferty, M. A. *Biochemistry* **11**, 1623–1633 (1972).
- Lauer, R. C., Solomon, P. H., Nakanishi, K. & Erlanger, B. F. *Experientia* **30**, 560–562 (1974).
- Laemmli, U. K. *Nature* **227**, 680–685 (1970).
- Spratt, T. E. & Kaiser, E. T. *J. Am. chem. Soc.* **106**, 6440–6442 (1984).
- Fersht, A. R. & Renard, M. *Biochemistry* **13**, 1416–1426 (1974).
- Habeeb, A. F. S. A. *Analyt. Biochem.* **14**, 328–336 (1966).
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. *J. biol. Chem.* **193**, 265–275.
- Dixon, G. *Biochemistry, J.* **55**, 170–172 (1953).

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Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element

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STEROID hormone receptors activate specific gene transcription by binding as hormone-receptor complexes to DNA enhancer elements termed hormone responsive elements (refs 1, 2 and references therein). A highly conserved 66-amino-acid region of the oestrogen and glucocorticoid receptors which corresponds to part of the receptor DNA-binding domain (region C) determines the specificity of target gene recognition^{3–5}. This region contains two subregions (CI and CII), encoded in two separate exons (refs 1, 7 and references therein), that are analogous to the 'zinc fingers' of the transcription factor TFIIIA (reviewed in ref. 6). The N-terminal CI finger determines the recognition specificity of the hormone responsive element⁸. A chimaeric oestrogen receptor, in which the CI finger is replaced with the corresponding glucocorticoid receptor CI finger region, activates transcription from a reporter gene containing a glucocorticoid-responsive element, but not from a reporter gene containing an oestrogen-responsive element⁸. We report here that three amino acids located at the C-terminal side of the oestrogen receptor CI finger play a key part in this specificity.

In the reporter gene *vit-tk-CAT* (ref. 9), the 5'-flanking region of the *Xenopus* vitellogenin A2 gene (*vit*), which contains a consensus palindromic oestrogen-responsive element (ERE), is inserted upstream of the herpes simplex virus promoter for thymidine kinase (*tk*). In the mouse mammary tumour virus (MMTV) reporter gene MMTV-CAT, the glucocorticoid-

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- Pollack, S., Jacobs, J. & Schultz, P. G. *Science* **234**, 1570–1573 (1986).
- Tramantano, A., Janda, K. D. & Lerner, R. A. *Science* **234**, 1566–1570 (1986).
- Jacobs, J., Schultz, P. G., Sugawara, R. & Powell, M. J. *Am. chem. Soc.* **109**, 2174–2176 (1987).

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responsive element (GRE) in the long terminal repeat of MMTV is positioned upstream of the *CAT*-indicator gene. It can be seen from Figs 1 and 2 that co-transfection of HeLa cells with the wild-type human oestrogen receptor (hER) expression vector HEO resulted, as expected, in the activation of *vit-tk-CAT* expression (but not of MMTV-*CAT* expression) in the presence (+) of oestradiol; MMTV-*CAT* expression, however, was specifically stimulated by the human glucocorticoid receptor (hGR) expression vector HGO in the presence (+) of dexamethasone. Note that essentially the same variations in target gene specificities were observed for the various receptor mutants studied below when a reporter containing a consensus GRE (GRE-*tk-CAT*, see ref. 8) was used as a glucocorticoid receptor (GR) reporter instead of MMTV-*CAT* (data not shown).

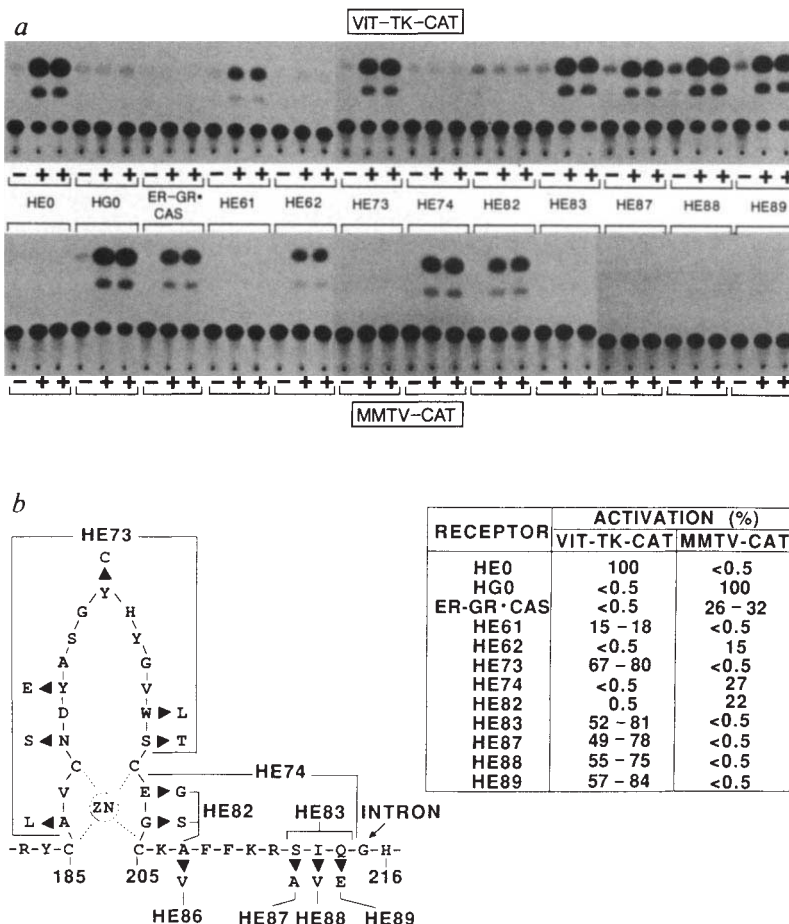
In agreement with our earlier results, the chimaeric receptors ER-GR.CAS (refs 3, 4) and HE62 (ref. 8) (in which the 66-amino-acid core and the CI N-terminal finger of region C of HEO were respectively replaced with the corresponding regions of hGR) activated the expression of MMTV-*CAT* but not of *vit-tk-CAT* (Fig. 1a and b). Conversely, HE61 (ref. 8) (in which the CII subregion of HEO was replaced with that of hGR) activated expression of *vit-tk-CAT*, but not of MMTV-*CAT*. To further delineate the amino acids responsible for this change of specificity, two new hER mutants were created in region CI of HEO. In HE73 (Fig. 1b), the N-terminal side (amino acids 185 to 188) and the finger tip (amino acids 189 to 201) were changed from the amino acids of hER to those of hGR, and in HE74 the C-terminal side amino acids 202 to 215 were changed (amino acid changes indicated by arrowheads in Fig. 1b; see also Fig. 4). HE73 retained the oestrogen-receptor (ER) specificity, whereas HE74 acquired the ability to activate MMTV-*CAT* but completely lost its capacity to activate *vit-tk-CAT* (Fig. 1a and

b). Further mutagenesis demonstrated that the change in target gene specificity observed with HE74 was essentially due to the replacement of three amino acids. Substitution of only these three discriminating amino acids (HE82) causes a complete change of specificity (compare HE82, HE83, HE87, HE88 and HE89 in Fig. 1a and b).

To investigate the individual role of these three discriminating residues, a series of single amino-acid substitutions was made. Each single substitution clearly decreased the efficiency of the corresponding proteins to act as ERs to a similar extent (HE84, HE85, HE86; Fig. 2a and b), but their target gene specificity was not changed. (Note, however, that HE84 slightly stimulated MMTV-*CAT*.) In contrast, a change in specificity was observed when substituting pairs of the discriminating amino acids with the corresponding GR residues (HE90, HE91, HE92 in Fig. 2a and b). The two combinations that involve the Glu to Gly (E → G) substitution (HE90 and HE91) resulted in a loss of ER function and a concomitant appearance of GR target gene specificity; this was particularly marked in HE91 (E, A → G, V). But HE92, in which the ER Glu203 was unchanged, recognized both ER and GR target genes, albeit with limited activating capacity. Note that all receptor mutants were present in about the same amount in the transfected HeLa cells, as judged from western immunoblotting (Fig. 3 and data not shown), and that the observed variations in reporter gene activation were therefore not due to differences in synthesis or stability of the mutated receptors.

Our results establish that three amino acids are of key importance to the ability of the ER to discriminate between an ERE and a GRE. Interestingly, these three amino acids are not situated in the tip of the CI finger, but on its carboxyl side. It has been proposed that this side of the zinc finger may form an

FIG. 1 Target gene specificity of hER mutants containing hER to hGR amino-acid substitutions in the DNA-binding domain subregion CI. a, The expression vectors HEO (ref. 4), HGO (ref. 4), ER-GR.CAS (ref. 3), HE61 (ref. 8), HE62 (ref. 8), HE73, HE74, HE82, HE83, HE87, HE88 and HE89 were co-transfected into HeLa cells with either the oestrogen-responsive *vit-tk-CAT* or the glucocorticoid-responsive MMTV-*CAT* reporter genes. Oestradiol (10^{-8} M) was added when indicated (+) to transfections with HEO and its mutant derivatives and dexamethasone (10^{-7} M) was added to transfections with HGO. b, The CI region of hER (amino acids 185 to 215; one-letter terminology) with its potential 'zinc finger' is represented and the position of the second intron of the hER gene is indicated. The positions of the amino-acid substitutions are indicated by an arrowhead pointing to the corresponding amino acid in hGR. The extent of the mutated region in each recombinant is defined by thin lines. Single average values ($\pm 20\%$) for the transcriptional activation of the two reporter genes (relative to either HEO or HGO, taken as 100%) result from three or more independent transfections with at least two different plasmid preparations. When two values are given, they correspond to two independent transfections with two different plasmid preparations. *Vit-tk-CAT* and MMTV-*CAT* expression were stimulated about 50- and 200-fold by HEO and HGO respectively. METHODS. HeLa cells at ~40% confluence were co-transfected with 1 μ g receptor expression vector, 1 μ g *vit-tk-CAT* or MMTV-*CAT* reporter gene and 3 μ g pCH110 (Pharmacia; a β -galactosidase expression vector used as a reference gene to normalize for variations in transfection efficiency). Transfection, preparation of cell extracts and quantitative determination of CAT activity have been described⁸. Mutagenesis of hER to generate HE73, HE74, HE82, HE83, HE87, HE88 and HE89 was essentially as described⁸, except that we used hER cloned into the *EcoRI* site in pSG5 (ref. 19) instead of that in pSG1. The sequences of the synthetic oligonucleotides used for site-directed mutagenesis are available on request.



α -helix that interacts with the DNA major groove, so it could be responsible for specific recognition of the cognate DNA sequence (refs 8, 10, 11 and references therein). Indeed, a putative α -helix motif located on the carboxyl side of the zinc finger in the DNA-binding domain of the yeast LAC9 regulatory protein may be largely responsible for the binding specificity¹².

The consensus palindromic ERE and GRE, to which the corresponding receptors bind as dimers⁵, differ by only two base pairs in each stem of the palindrome (ERE: TGACC versus GRE: TGTTT) (refs 1, 13, 14 and references therein). It is likely that the three amino acids characterized here are central to the discrimination between these two base pairs. Other amino acids conserved between the ER and GR CI subregion (see Fig. 4) are probably important either for specific interactions with the conserved bases of the palindromic elements or for non-specific interactions with the DNA backbone (see ref. 8 for a discussion of the possible role of the CII subregion in the overall efficiency of receptor binding to its responsive element (RE)). That only two amino-acid substitutions (see mutant HE91 in Fig. 2) are required for the discrimination between two related REs, readily indicates that new receptors and REs might have co-evolved from their respective ancestors (note also the intermediate case of mutant HE92). Also, one would predict that nuclear receptors which share the same three discriminating amino acids should be able to activate the same RE. Comparison of the amino-acid sequences of eight nuclear receptors (see Fig. 4) shows that the amino-acid sequence of the carboxyl side of the CI finger is

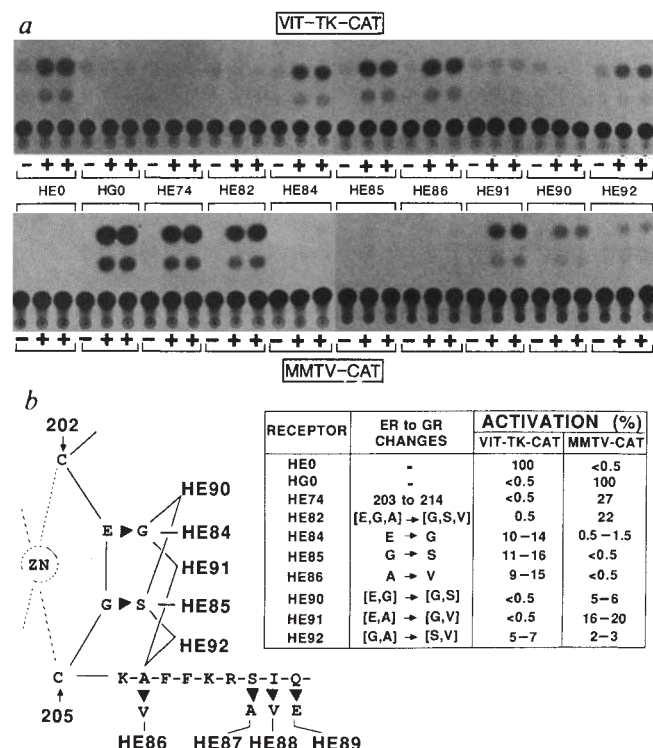


FIG. 2 Target-gene specificity of hER mutants with amino acids 203, 204 and/or 207 changed to their GR homologues. *a*, Assay for CAT activity after transfection into HeLa cells of the expression vectors HE0, HG0, HE74, HE82, HE84, HE85, HE86, HE90, HE91 and HE92, together with either the *vit-tk-CAT* or *MMTV-CAT* reporter genes. Oestradiol and dexamethasone were added as described in Fig. 1*a* legend. *b*, The position of the amino-acid substitutions is as indicated in Fig. 1*b*. The transcriptional activation of the two reporter genes by the various hER mutants was expressed relative to HE0 and HG0, as indicated in Fig. 1*b*. Mutagenesis of hER to generate HE84, HE85, HE86, HE90, HE91 and HE92 and all other methods were performed as described in the legend to Fig. 1, except that cell-extract aliquots corresponding to five-times less β -galactosidase activity were used for CAT assays in the case of HE0 and HG0 transfections.

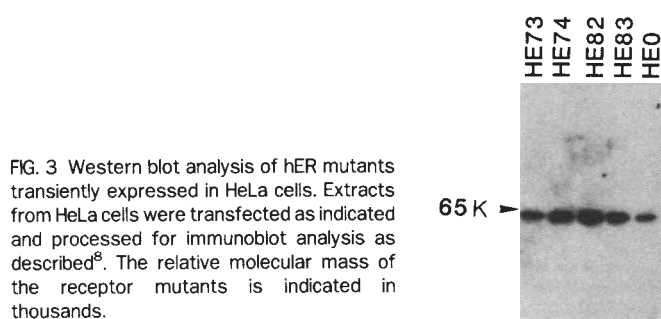


FIG. 3 Western blot analysis of hER mutants transiently expressed in HeLa cells. Extracts from HeLa cells were transfected as indicated and processed for immunoblot analysis as described⁸. The relative molecular mass of the receptor mutants is indicated in thousands.

remarkably conserved, with the striking exception of the three discriminating amino acids (indicated by arrows in Fig. 4); two subfamilies can be identified (Fig. 4*a* and *b*). The four members of one of these subfamilies (Fig. 4*a*) are known to recognize a common RE (refs 15, 16 and references therein). Moreover, it has recently been shown (ref. 17; H. de V., D. Metzger and P.C., manuscript in preparation) that two members of the second subfamily (Fig. 4*b*) which share the same three discriminating amino acids (retinoic acid receptor and thyroid hormone receptor, RAR and T3R respectively) may recognize a common palindromic RE, which is related to the ERE. (Considering the sequence similarities, it is probable that the RE of the vitamin D3 receptor is also close to that of RAR and T3R.) Interestingly, we have recently found (H. de V., D. Metzger and P.M., manuscript in preparation) that both T3R and RAR, which share two out of the three discriminating amino acids with the ER, can activate transcription of a reporter gene containing an ERE (but not a GRE), albeit with a lower efficiency than the ER (see also ref. 18 for an account of a weak stimulation of *vit-tk-CAT* by RAR). This observation is consistent with the properties of HE86, which contains the two discriminating amino acids common to ER, T3R and RAR, and specifically activates *vit-tk-CAT*, although with less efficiency than the wild-type hER (HE0) (Fig. 2).

Finally, it should be stressed that our data do not exclude the possibility that additional amino acids (such as some of those located in the finger tip) might help in the discrimination between an ERE and a GRE, although the amino acids we have identified here are sufficient. In this respect we note that HE82 is significantly less efficient than HG0 in the activation of MMTV-CAT (Fig. 1), and that its affinity for a GRE *in vitro* is lower than that of the GR (unpublished data). Additional

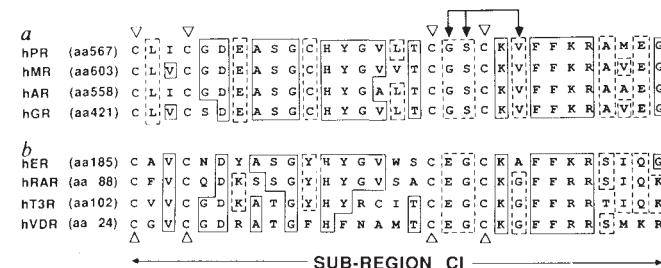


FIG. 4 Comparison of the amino-acid sequences of the subregion CI (N-terminal 'zinc finger') of the DNA-binding domain (region C) of various members of the human nuclear receptor family. The conserved cysteine residues that may tetrahedrally coordinate zinc to form a 'zinc finger' (see Fig. 1*b*) are indicated by open arrowheads. The arrows point to the position of the three 'discriminating' amino acids (see text). Amino acids which are common to only one of the two subfamilies (*a*, hPR, hMR, hAR and hGR; *b*, hER, hRAR, hT3R and hVDR; see text) are boxed with dashed lines, whereas those common to the two subfamilies are boxed with solid lines. The receptors hPR (ref. 20), hMR (ref. 16), hAR (ref. 21), hGR (ref. 22), hER (ref. 23), hRAR (refs 18 and 24), hT3R (ref. 25) and hVDR (ref. 26) are the progesterone, mineralocorticoid, androgen, glucocorticoid, oestrogen, retinoic acid, thyroid hormone and vitamin D3 receptors respectively.

mutagenesis aimed at identifying such possible helper amino acids is in progress. □

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- Green, S. & Chambon, P. *Trends Genet.* **4**, 309–314 (1988).
- Evans, R. M. *Science* **240**, 889–895 (1988).
- Green, S. & Chambon, P. *Nature* **325**, 75–78 (1987).
- Kumar, V. *et al.* *Cell* **51**, 941–951 (1987).
- Kumar, V. & Chambon, P. *Cell* **55**, 145–156 (1988).
- Klug, A. & Rhodes, D. *Trends biochem. Sci.* **12**, 464–469 (1987).
- Ponglikitmongkol, M., Green, S. & Chambon, P. *EMBO J.* **7**, 3385–3388 (1988).
- Green, S., Kumar, V., Theulaz, I., Wahli, W. & Chambon, P. *EMBO J.* **7**, 3037–3044 (1988).
- Klein-Hitpaß, L., Schorpp, M., Wagner, U. & Ryffel, G. U. *Cell* **46**, 1053–1061 (1986).
- Berg, J. M. *Proc. natn. Acad. Sci. U.S.A.* **85**, 99–102 (1988).
- Gibson, T. J., Postma, J. P. M., Brown, R. S. & Argos, P. *Protein Engng* **2**, 209–218 (1988).
- Witte, M. M. & Dickson, R. C. *Molec. cell. Biol.* **8**, 3726–3733 (1988).
- Klock, G., Strähle, U. & Schütz, G. *Nature* **329**, 734–736 (1987).
- Martinez, E., Givel, F. & Wahli, W. *EMBO J.* **6**, 3719–3727 (1987).
- Ham, J., Thompson, A., Needham, M., Webb, P. & Parker, M. *Nucleic Acids Res.* **16**, 5263–5276 (1988).
- Arriza, J. L. *et al.* *Science* **237**, 268–275 (1987).
- Umesono, K., Giguère, V., Glass, C. K., Rosenfeld, M. G. & Evans, R. M. *Nature* **336**, 262–265 (1988).
- Petkovich, M., Brand, N. J., Krust, A. & Chambon, P. *Nature* **330**, 444–450 (1987).
- Green, S., Issemann, I. & Scheer, E. *Nucleic Acids Res.* **16**, 369 (1988).
- Misrahi, M. *et al.* *Biochem. Biophys. Res. Commun.* **143**, 740–748 (1987).
- Chang, C., Kokontis, J. & Liao, S. *Proc. natn. Acad. Sci. U.S.A.* **85**, 7211–7215 (1988).
- Hollenberg, S. M. *et al.* *Nature* **318**, 635–641 (1985).
- Green, S. *et al.* *Nature* **320**, 134–139 (1986).
- Brand, N. *et al.* *Nature* **332**, 850–853 (1988).
- Weinberger, C. *et al.* *Nature* **324**, 641–646 (1986).
- Baker, A. R. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **85**, 3294–3298 (1988).

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Fertile transgenic rice plants regenerated from transformed protoplasts

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THE generation of transgenic plants using gene transfer techniques is important to both the investigation of gene regulation and the genetic engineering of crops¹. The Ti plasmid of *Agrobacterium tumefaciens* is now routinely used to transform dicotyledonous plants², and the transfer of foreign genes to unorganized tissue^{3–6} and plants^{7,8} has been accomplished using direct DNA transfer methods^{9–11}. A protocol for the easy and reproducible production of fertile transgenic cereals, however, has not yet been described. We report here the production of fertile transgenic rice plants obtained by introducing the bacterial *hph* gene, encoding hygromycin B resistance¹² (Hm^r), into protoplasts of *Oryza sativa* (L.) by electroporation. The non-selectable gene encoding β -glucuronidase was also transferred with the *hph* gene and its expression was detected in the progeny of the stable transformant.

The kanamycin resistance (Km^r) gene coding for neomycin phosphotransferase II (NPT II) is often used as a selectable marker to produce transgenic plants¹. We found, however, that the bacterial *hph* gene is a more effective marker for the selection of stable transformants from embryogenic rice protoplasts and on the basis of this we chose, as an integration vector, plasmid pGL2, which carries the *hph* gene flanked by the 35S promoter and a polyadenylation site from cauliflower mosaic virus¹³ (CaMV) (Fig. 2a).

Because embryogenic protoplasts of rice are small (10–20 μ m in diameter), electroporation was carried out using a long pulse (10 ms), generated by a large capacitor (800 μ F) and a moderate voltage (300 V cm⁻¹). Under these conditions, the plating

efficiency (0.2–1.2%, Table 1) was 10–20% of that of non-electroporated control protoplasts grown by the nurse culture method¹⁴. No modification of the original protocol was required for the culture of electroporated protoplasts. After 2–3 weeks of selection with hygromycin B (20 μ g ml⁻¹), resistant colonies became clearly visible (Fig. 1a). Little background colony growth was observed in control experiments in which hygromycin B (20 μ g ml⁻¹) was added to the R2 medium¹⁵ 2 weeks after electroporation.

The frequency of resulting Hm^r clones depended on the protoplast preparation and the cuvettes used for electroporation (Table 1). In all the experiments, the transformation frequencies fell in the range 0.1–0.6% of clones subjected to selection. This range is lower than those obtained for the recovery of Km^r clones after the electroporation of maize protoplasts^{5,8} and the treatment of non-embryogenic rice protoplasts with polyethylene glycol (PEG)⁶.

Most of the Hm^r colonies continued to grow after transferring them to a solid N6 medium¹⁶ containing the same concentration of hygromycin B. Three to four weeks later, the Hm^r calli were transferred to an N6 regeneration medium¹⁴ containing no hygromycin B. Within 3–6 weeks, shoots as well as roots became visible. The efficiency of regeneration is evident from the fact that more than 50% of the Hm^r calli yielded shoots in every experiment (Fig. 1b).

The presence of the *hph* gene on the chromosomes of stable transformants was shown by a Southern blot analysis of the plants regenerated from Hm^r calli (Fig. 2a–c). After the digestion of genomic DNA with *Bam*H1, six plants from four different experiments were found to contain a 1.1-kilobase (kb) fragment that hybridized to the *hph* gene (lanes 3–8). The detection of multiple bands, after digestion with *Eco*RV (lanes 9–11), indicated the presence of multiple chromosomal integration sites. By using a mixed probe containing both the *hph* probe and a single copy gene probe, the copy number of the integrated *hph* gene was estimated to be 2–10 per diploid cell (data not shown).

Plantlets regenerated from Hm^r calli were transferred to pots where they grew to maturity, flowered and set seeds (Fig. 1c). From fourteen such plants, we chose four, plants 3-1, 6-1, 6-3 and 8-1, to study the transmission of the *hph* gene. Thus, selfed seeds were subjected to hygromycin B at a concentration (30 μ g ml⁻¹) previously found to be sufficient to inhibit the germination of control seeds. The subsequent survival of selfed seeds showed that the active *hph* gene had been transmitted to the progeny (Fig. 1d; Table 2).

Further evidence for this transmission was obtained by germinating selfed seeds in the absence of hygromycin B and analysing their DNAs by Southern hybridization (Fig. 2c). Among ten progeny from the plant 6-3, seven contained the *hph* gene. Similarly, among nine progeny from the plant 8-1, six contained sequences hybridizing the *hph* gene. The two bands which hybridized the *hph* gene were found to co-segregate in the progeny of the plant 8-1. These results indicate that either a single insert or multiple inserts of the functional *hph* gene are present on the chromosomes of the primary transformants. To clarify the mode of transmission of the integrated *hph* gene, selfed and backcross progeny of other transformants are now being investigated.

TABLE 2 Transmission of the Hm^r phenotype to progeny

Seed	Total no. seeds tested	No. resistant seeds found	No. sensitive seeds detected
Control	96	0	96
3-1 self	40	38	2
6-1 self	95	85	10
6-3 self	29	25	4
8-1 self	45	31	14

The methods for testing the Hm^r phenotype are described in Fig. 1d.

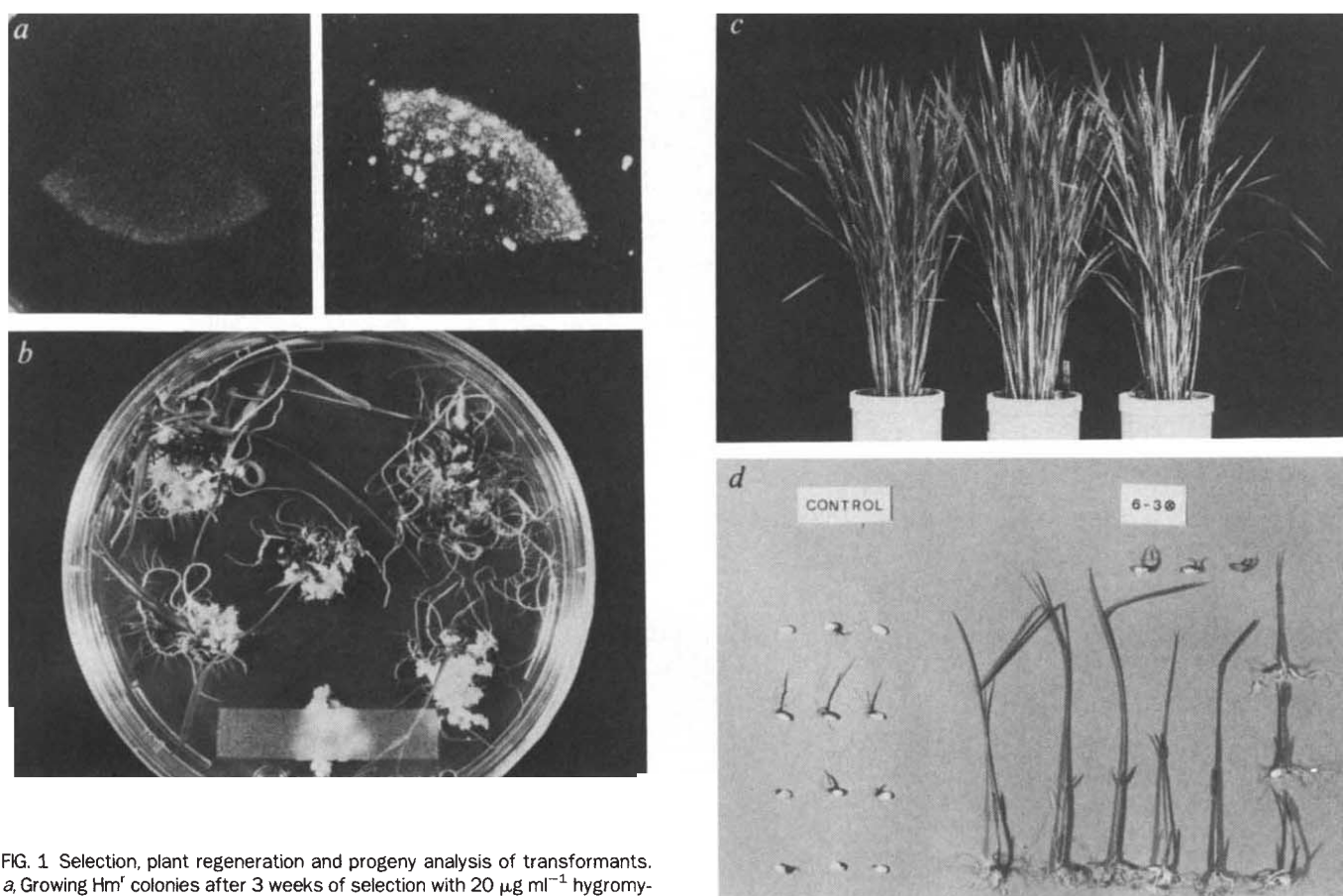


FIG. 1 Selection, plant regeneration and progeny analysis of transformants. *a*, Growing Hm' colonies after 3 weeks of selection with $20 \mu\text{g ml}^{-1}$ hygromycin B (5 weeks after electroporation). Protoplasts were electroporated in the presence of plasmid DNA and carrier DNA (right); control culture electroporated with carrier DNA alone (left). *b*, Regeneration of shoots and roots from Hm' calli. *c*, Fertile plants obtained from Hm' calli. *d*, Test of the Hm' phenotype in the control seeds (left) and the progeny of the transformant 6-3 (right). **METHODS.** Protoplasts were isolated from 1–2-month-old suspension cultures derived from mature embryo calli of *cv. Nipponbare* as described previously¹⁴. The protoplasts were washed twice and resuspended (4×10^6 protoplasts ml^{-1}) in a buffer containing 70 mM KCl, 5 mM MgCl_2 , 0.1% MES, pH 5.8 and 0.4 M mannitol. Aliquots (0.5 ml) of the protoplast suspension were mixed with the supercoiled pGL2 plasmid DNA ($10 \mu\text{g ml}^{-1}$) and carrier DNA ($50 \mu\text{g ml}^{-1}$). Carrier DNA was prepared by dissolving calf thymus DNA (Sigma) in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 and sterilized using a disposable filter. After the incubation of the protoplast-DNA mixture in ice for 5 min, a sample (0.5 ml) was transferred to a precooled plastic cuvette and electroporated by a capacitor-discharge system (X-cell 450, Promega). After 10 min at 4°C , protoplasts were mixed with 0.5 ml of the molten agar R2 medium¹⁵ contain-

ing 2.5% agarose (Sea Plaque, FMC) in a plastic plate (35 mm in diameter). After cooling, the agarose was cut into four blocks, which were transferred to a plastic plate (6 cm in diameter) containing 5 ml of the R2 medium and cultured by the nurse culture method¹⁴. Hygromycin B (Sigma) was added to the medium 2 weeks later at a final concentration of $20 \mu\text{g ml}^{-1}$. Agarose blocks containing growing colonies were transferred 2–3 weeks later to an N6 soft agar medium¹⁶ containing $20 \mu\text{g ml}^{-1}$ hygromycin B and incubated for 1–2 weeks. Individual colonies were then transferred to the same N6 medium with increased agarose concentration (0.5%). When the calli were ~5 mm in diameter, they were transferred to the regeneration medium. Plant regeneration was as described previously¹⁴. For analysis of Hm' in progeny, selfed seeds were sterilized in 0.6% NaClO_4 , washed twice in sterile distilled water and placed onto MS medium¹⁹ containing $30 \mu\text{g ml}^{-1}$ hygromycin B. They were incubated for 2 weeks at 25°C under light.

TABLE 1 Frequency of Hm' clones

Experiment	Cuvette	pGL2 ($\mu\text{g ml}^{-1}$)	No. protoplasts used ($\times 10^6$)	No. clones tested for Hm' ($\times 10^3$)	No. Hm' clones found	Frequency of stable transformations (%)
I	1	0	2	10.5	0	0
	2	10	2	14.3	65	0.45
	3	10	2	14.5	58	0.40
	4	10	2	11.6	75	0.65
	5	10	2	13.7	70	0.50
	6	10	2	12.4	80	0.60
II	1	0	2	17.8	0	0
	2	10	2	24.0	32	0.13
	3	10	2	18.0	27	0.15
	4	10	2	4.8	12	0.25

Experiments I and II consisted of several electroporation trials, each of which was performed with a cuvette, using protoplasts derived from one preparation. Colonies subjected to selection for Hm' were counted 12 days after electroporation. The number of Hm' clones was scored after 3 weeks of culture in the presence of $20 \mu\text{g ml}^{-1}$ hygromycin B.

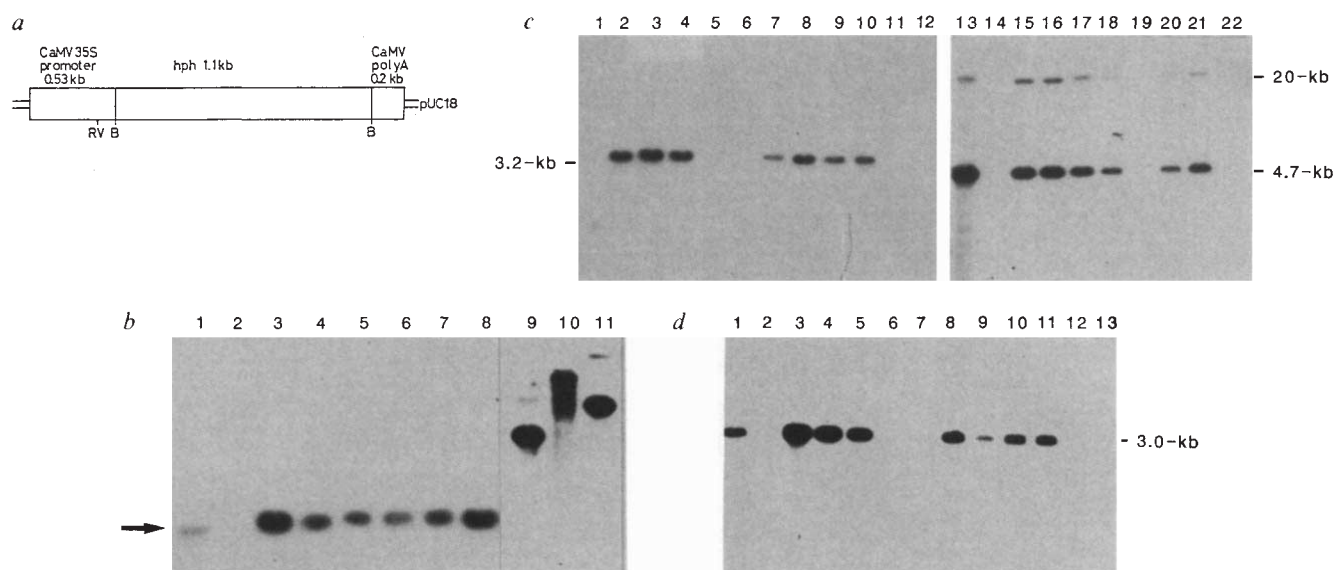


FIG. 2 Molecular confirmation of the chromosomal integration of the foreign genes. *a*, Diagram of pGL2. pGL2 is a pUC18 derivative carrying the 35S promoter and a polyadenylation site of CaMV (ref. 13) together with the *Bam*H1 segment containing the *hph* gene derived from pLG88 (ref. 20). It contains a unique *Eco*RV site within the 35S promoter region: B, *Bam*H1; RV, *Eco*RV. *b-d*, Southern blot analysis of the genomic DNA from primary transformants and their progeny. *b*, Genomic DNA from nine independent *Hm*^r plants digested with *Bam*H1 or *Eco*RV. Lane 1, 1.1-kb *Bam*H1 fragment of pGL2 containing the *hph* gene indicated by arrow; lane 2, DNA from non-transformed plants; lanes 3–8, *Bam*H1-digested DNA from 6 independent *Hm*^r plants; lane 9–11, *Eco*RV-digested DNA from three *Hm*^r plants. *c*, DNA from progeny of transformants 6-3 and 8-1 digested with *Eco*RV. Lane 1, DNA from non-transformed plants; lane 2, DNA from the transformant 6-3; lanes 3–12, DNA from 10 progeny of the transformant 6-3; lane 13, DNA from the transformant 8-1; lanes 14–22, DNA from nine progeny of the plant 8-1. *d*, DNA from the GUS transformant G1 and its progeny digested with *Eco*RI and *Hind*III. Lane 1, pBI221 digested with *Eco*RI and *Hind*III; lane 2, DNA from non-transformed plants; lane 3, DNA from the primary transformant G1; lanes 4–13, DNA from 10 progeny. *e*, Expression in the seed of the GUS marker co-transformed with pGL2. Left, control seed; right, progeny seed expressing GUS activity. METHODS. DNA samples were prepared from transgenic plants according to a published protocol²¹. For Southern blot analysis²², 1–2 µg of digested DNA

was electrophoresed in a 1.0% agarose gel, transferred to a nylon membrane (Bio-Rad) and the blots were hybridized with the ³²P-labelled²³ 1.1-kb *Bam*H1 fragment of pGL2 corresponding to the *hph* gene (*b*, *c*) or 1.9-kb *Xba*I-*Sst*I fragment of pBI221 corresponding to the GUS gene (*d*). The *Bam*H1 fragment of pGL2 (10 µg) (*a*) or 12 µg of pBI221 digested with *Eco*RI and *Hind*III (*c*) was electrophoresed as a marker. For cotransformation of the GUS marker with the *hph* gene, 20 µg ml⁻¹ of pBI221 was mixed with 10 µg ml⁻¹ of pGL2 and 50 µg ml⁻¹ of carrier DNA and electroporated as described in Fig. 1. For histochemical staining of the GUS activity, seeds were cut into halves and stained with X-gluc according to ref. 24.

A preliminary experiment to examine co-transformation of an unlinked, non-selectable gene was performed using the β -glucuronidase (GUS) gene under the control of the CaMV 35S promoter and the 3' region of the nopaline synthetase gene¹⁷. The addition of this GUS plasmid (20 µg ml⁻¹) did not influence the plating efficiency or the frequency of *Hm*^r colonies. The selfed seeds of one of the *Hm*^r plants that showed GUS activity in their leaf extracts, referred to as G1, were subjected to further analyses. Southern blot analysis of G1 and its progeny showed that 7 out of 10 progeny plants contained the complete 3.0-kb GUS gene (Fig. 2*d*). The progeny seeds were individually cut into two halves and stained with 5-bromo-4-chloro-3-indolylglucuronide (X-gluc) to detect GUS activity. Whereas no activity was detected in 20 untransformed control seeds, 41 out of 57 transformed seeds were found to have GUS activity in the embryo and the endosperm (Fig. 2*e*), indicating that the GUS gene, integrated in the primary transformant, is stably inherited by progeny.

These results demonstrate that the co-transformation of a non-selectable gene with a selectable marker can be used to generate fertile transgenic rice. It seems that the construction of a plasmid carrying both the selectable marker and the non-selectable gene is not a prerequisite for the successful transfer of the latter, and that, as observed in the case of tobacco¹⁸,

co-transformation is a simple alternative method for generating transgenic plants carrying active genes. □

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- Schell, J. S. *Science* **237**, 1176–1183 (1987).
- Cocking, E. C. & Davey, M. R. *Science* **236**, 1259–1262 (1987).
- Potrykus, I. *et al. Molec. gen. Genet.* **199**, 161–168 (1985).
- Lörz, H., Baker, B. & Schell, J. *Molec. gen. Genet.* **199**, 178–182 (1985).
- Fromm, M., Taylor, L. P. & Walbot, V. *Nature* **319**, 791–793 (1986).
- Uchimiya, H. *et al. Molec. gen. Genet.* **205**, 461–468 (1986).
- de la Peña, A., Lörz, H. & Schell, J. *Nature* **325**, 274–276 (1987).
- Rhodes, C. A., Pierce, D. A., Mettler, I. J., Mascarenhas, D. & Detmer, J. *Science* **240**, 204–207 (1988).
- Davey, M. R. *et al. Pl. Sci. Lett.* **18**, 307–313 (1980).
- Krens, F. A., Molendijk, L., Wullem, G. L. & Schilperoort, R. A. *Nature* **296**, 72–74 (1982).
- Paszkowski, J. *et al. EMBO J.* **3**, 2717–2722 (1984).
- Gritz, L. & Davies, J. *Gene* **25**, 179–188 (1983).
- Pietrzak, M., Shillito, R. D., Hohn, T. & Potrykus, I. *Nucleic Acids Res.* **14**, (1986).
- Kyozuka, J., Hayashi, Y. & Shimamoto, K. *Molec. gen. Genet.* **206**, 408–413 (1987).
- Ohira, K., Ojima, K. & Fujiwara, A. *Pl. Cell Physiol. Tokyo* **14**, 1113–1121 (1973).
- Chu, C. C. *et al. Scientia. sin.* **16**, 659–688 (1975).
- Jefferson, R. A., Kavanagh, T. A. & Bevan, M. W. *EMBO J.* **6**, 3901–3907 (1987).
- Schocher, R. J., Shillito, R. D., Saul, M. W., Paszkowski, J. & Potrykus, I. *Bio/Technology* **4**, 1093–1096 (1986).
- Murashige, T. & Skoog, F. *Physiologia Pl.* **15**, 473–497 (1962).
- Blochinger, K. & Diggelmann, H. *Molec. cell. Biol.* **4**, 2929–2931 (1984).
- Walbot, V. & Warren, C. *Molec. gen. Genet.* **211**, 27–34 (1988).
- Southern, E. M. *J. molec. Biol.* **98**, 503–517 (1975).
- Feinberg, A. P. & Vogelstein, B. *Analyt. Biochem.* **132**, 6–13 (1983).
- Jefferson, R. A. *Pl. molec. Biol. Rep.* **5**, 387–405 (1987).

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Genomic footprinting with *Taq* polymerase

H. P. Saluz and J. P. Jost

The linear amplification of chemically sequenced DNA with DNA polymerase from *Thermus aquaticus* permits the direct detection of DNA methylation and protein–DNA interaction sites *in vivo*.

DNA methylation and protein–DNA interactions play a crucial role in gene regulation^{1,2}. Genomic sequencing — pioneered by Church and Gilbert³, and further refined in our laboratory⁴ — enables the study of the methylation state of any gene in a strand- and sequence-specific manner.

When used to investigate areas of protein/DNA interaction, the technique is referred to as genomic footprinting. In this technique, cells in suspension are treated with dimethylsulphate (DMS) or ultraviolet light to modify specific DNA bases, which undergo a quantitative cleavage reaction (β -elimination)^{5,7}. The modified sites are then identified by genomic sequencing. Proteins bound to DNA protect these areas from modification by DMS and subsequent chemical cleavage, leaving their “footprint” on the DNA. In contrast, ultraviolet light cleaves at sites of protein–DNA interaction.

Despite the potential of *in vivo* footprinting for revealing protein–DNA interactions and genomic sequencing for detecting modifications to the DNA's native state, the current procedure is rather time-consuming and complex. We sought therefore to simplify the technique and make it more accessible to less experienced users⁸. A flow diagram of our new method is shown in Fig. 1a.

Abbreviated strategy

The first steps of both the old method and our new technique are the same: total genomic DNA is digested with a suitable restriction enzyme, and the resulting DNA fragments are chemically sequenced as described by Maxam and Gilbert⁹, Fritzsche *et al.*¹⁰, or Rubin and Schmid¹¹. But in our technique, sequencing is followed by selective, linear amplification with the thermostable DNA polymerase from *Thermus aquaticus*, using a synthetic primer labelled to a very high specific radioactivity. The products of the linear amplification (Fig. 2) are then run on a sequencing gel, and the sequence information is directly obtained by exposing the dried gel to an X-ray film.

A step forward

The new technique permits sequence information to be gathered for any gene, regardless of its copy number and the size of the total genome, without electroblotting or hybridization steps (Fig. 1b). Moreover, as restriction enzymes are used only to reduce the viscosity of the genomic

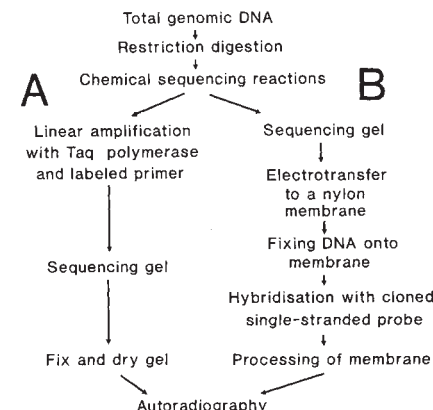


FIG. 1 Flow diagram for performing genomic experiments using the new expedited procedure (a), and the classical method of genomic sequencing (b).

DNA in the reaction mixture, less expensive endonucleases can be used, as long as they do not cleave within the target sequence.

But the success of our technique is highly dependent upon the purity of the *Taq* polymerase used, which can vary from batch to batch. The recent commercial availability of recombinant *Taq* polymerase should eliminate this problem. [Editor's note: Recombinantly produced *Taq* polymerase can be purchased from Perkin-Elmer Cetus and United States Biochemical Corporation. Purified natural *Taq* is sold by Perkin-Elmer Cetus, Stratagene, BRL, Beckman Instruments, and Promega.]

Our simplified procedure for studying the native state of genomic DNA relies on the linear amplification of cleaved DNA

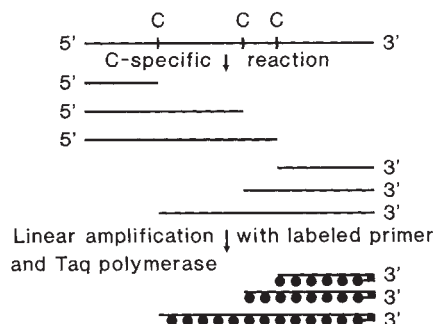


FIG. 2 A diagram illustrating the linear amplification of the sequencing reaction products. Restriction enzyme-digested DNA is first subjected to chemical sequencing reactions. The fragments generated by β -elimination are then annealed to labelled oligonucleotide primers (black squares), and the complementary strand (dotted line) is synthesized by *Taq* polymerase.

fragments to produce many radioactively labelled copies. The resulting increase in sensitivity allows the more rapid detection of single-copy mammalian genes, and should permit the investigation of such complex genomes as those of higher plants. The technique retains all of the benefits of the original genomic sequencing procedure. The unique thermostability of *Taq* polymerase permits the optimal annealing of the primer to the denatured target template, and its faithful elongation. But so far, procedures for studying DNA methylation are still based on the detection of chemical cleavage products. 5-Methylcytosine reacts poorly with hydrazine, resulting in a lack of cleavage upon alkaline treatment. The position of the 5-methylcytosine is then revealed by a gap in the sequence ladder.

Because chemical cleavage of the DNA results in fragments of different lengths, it is only possible to amplify each fragment linearly. But if procedures were available quantitatively to deaminate 5-methylcytosine residues to produce thymidine, the modified DNA could be amplified exponentially by the popularly used symmetrical amplification procedure of the polymerase chain reaction (PCR)^{12,13}. Following dideoxy sequencing, the position of the original 5-methylcytosine would be detected by the presence of an A-T pair instead of a G-C pair in the undeaminated control DNA. We are now investigating this approach, because it would speed up the procedure enormously. □

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1. Razin, A. Cedar, H. & Riggs, A.D. *DNA Methylation*, 392 (Springer, New York, 1984).
2. Adams, R.L.P. & Burdon, R.H. *Molecular Biology of DNA Methylation*, 247 (Springer, New York, 1985).
3. Church, G.M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1991-1995 (1984).
4. Saluz, H.P. & Jost, J.P. *Gene* **42**, 151-157 (1986); *A Laboratory Guide to Genomic Sequencing*, 163 (Birkhäuser, Boston, 1987).
5. Becker, P.B., Ruppert, S. & Schütz, G. *Cell* **51**, 435-443 (1987).
6. Saluz, H.P., Feavers, I.M., Jiricny, J. & Jost, J.P. *Proc. natn. Acad. Sci. U.S.A.* **85**, 6697-6700 (1988).
7. Becker, M.M. & Wang, J.C. *Nature* **309**, 795-798 (1984).
8. Saluz, H.P. & Jost, J.P. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
9. Maxam, A.M. & Gilbert, W. *Meth. Enzym.* **65**, 499-560 (1980).
10. Fritzsche, E., Hayatsu, H., Igloi, G.L., Iida, S. & Kossel, H. *Nucleic Acids Res.* **15**, 5517-5528 (1987).
11. Rubin, C.M. & Schmid, C.W. *Nucleic Acids Res.* **8**, 4613-4619 (1980).
12. Saiki, R.K. *et al.* *Science* **230**, 1350-1354 (1985).
13. Erlich, H.A., Gelfand, D.H. & Saiki, R.K. *Nature* **331**, 461-462 (1988).

FASEB exposition in New Orleans

The best of what biology has to offer will be on display at next week's FASEB meeting in New Orleans, Louisiana — from a sponge for destaining electrophoresis gels to transgenic mice for cancer research.

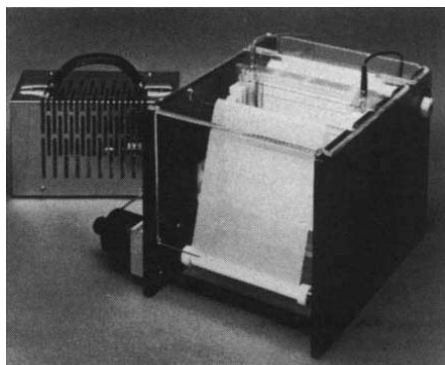
KONTRON Instruments sells an **ultra-centrifuge toolkit** that contains everything needed for the correct use of Kontron ultracentrifugation rotors, tubes and capping systems — packaged together in a black storage case (*Reader Service No. 101*). The toolkit includes a tube cap vise with inserts; tube, cap and adaptor removal tools; a torque wrench with sockets; a hex driver; various lubricants; and tube stands in a range of sizes. Kontron says the tools can extend the lifetime of ultracentrifuge rotors and capping systems, if used properly. The company also offers IBM-compatible centrifugal separation simulation software which allows users to predict the optimal conditions for performing a variety of separations using any standard rotor.

DuPont's transgenic OncoMouse — the world's first patented animal — is now on sale for use in cancer research (*Reader Service No. 102*). The **transgenic mouse** is reared by Charles River Laboratories, and contains the viral Harvey *ras* (v-Ha-ras) oncogene controlled by the mouse mammary tumour virus (MMTV) promoter. OncoMouse is intended to be used to study the pathogenesis of cancer, to screen chemopreventive and anticancer agents, and to test the carcinogenicity of compounds: 30 per cent of female mice develop breast cancer within 100 days. Both males and females develop parotid adenocarcinomas and harderian hyperplasia. DuPont also sells a line of monoclonals specific for the p21 protein product of v-Ha-ras for measuring oncogene expression. The price of each OncoMouse is \$50 (US) for orders under 25. The company plans to offer future strains of OncoMouse incorporating *ratneu* and *c-myc* oncogenes, under the control of other types of promoters.

Gel goodies

ICN Biomedicals has introduced an **electrophoresis destaining sponge** for leaching Coomassie Brilliant Blue stains out of electrophoresis gels (*Reader Service No. 103*). ICN Biomedicals says the sponge's high affinity for Coomassie reduces by half the number of methanol rinses required for destaining. The 5 × 5 cm sponges are supplied in packs of 50. The company says one sponge is sufficient for destaining a 10 × 10 × 0.1 cm gel, with no changes in the standard destaining procedure.

Hoefer Scientific has a new **sequencing apparatus** that separates and blots frag-



Up to 400 bases-a-run: Hoefer's TE2000.

ments in one operation (*Reader Service No. 104*). The model TE2000 direct blotting sequencer first separates fragments on a 14 × 16 cm gel. Fragments then migrate off the bottom of the gel onto a nylon membrane moving on a belt directly beneath the gel. The speed of the belt is adjustable, to eliminate the band crowding often seen at the top of sequencing gel blots. Hoefer says that the new sequencer/blotter can be used to sequence up to 400 bases in a single run, without handling fragile gels. The membrane can also be used to detect sequences by colourimetric detection, eliminating the need for working with radioactive isotopes.

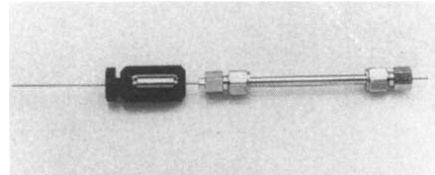
AT Biochem recently unveiled a new **electrophoresis polymer** as an alternative to agarose and polyacrylamide (*Reader Service No. 105*). The company will not disclose the composition of its Hydrolink polymer, pending approval of its patent. But AT Biochem says the Hydrolink matrix is less susceptible to breakage, produces sharper bands, is less toxic, and can accommodate ten times the loading levels of traditional gel media. The company sells formulations for both double-stranded DNA separations and sequencing gels. The Hydrolink media comes as a ready-to-pour liquid, with specially formulated buffers and denaturing reagents that eliminate the need for urea. The company plans to introduce a matrix for protein separation later this year.

Chromatographic analysers

Applied Biosystems has introduced an **amino acid analyser** that combines hydrolysis, derivatization and separation steps in one complete system (*Reader Service No. 106*). The model 420A amino acid analyser's hydrolyser unit breaks the bonds between amino acids to liberate free amino acids using hydrochloric acid at temperatures of greater than 1,000 °C.

The unit completely eliminates the need for time-consuming manual hydrolysis: researchers simply add the protein sample and return in less than an hour to collect results. The 420A's PTC derivatization unit labels free amino acids with a chemical which Applied Biosystems says allows detection at high sensitivities. Once derivatized, the amino acids are separated by liquid chromatography and detected and identified by comparison with a known standard.

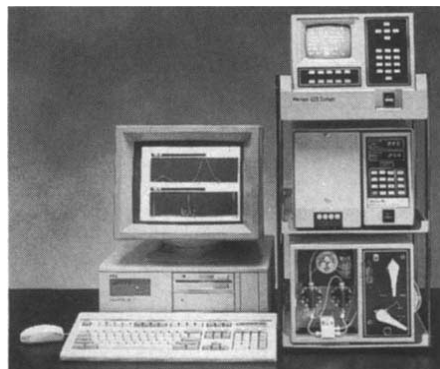
Hamilton has expanded its range of **reversed-phase columns** to include columns especially designed for analysis in protein and peptide matrices (*Reader Service No. 107*). Hamilton's new PRP-3 columns can be regenerated in an overnight process in order to eliminate residual



The Hamilton PRP-3: acid to alkaline.

protein buildup and to restore performance. The PRP-3 columns are available in pre-column, analytical and preparative sizes, and can be used over the pH range 1–12.

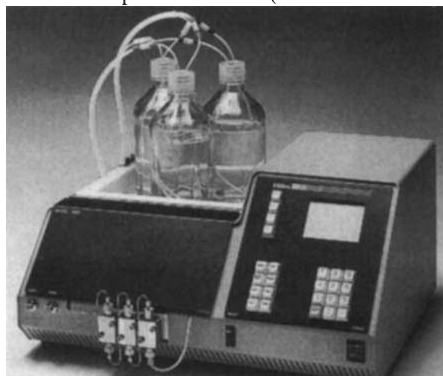
Waters recently introduced a liquid chromatography system designed specifically for bioseparations (*Reader Service No. 108*). The Waters model 625 **bio-separation chromatography system** incorporates non-metallic polymeric materials with low dispersion capabilities for micro-bore, analytical and micropreparative separations. The system can be used with ion exchange, affinity, hydrophobic interaction, gel filtration and reverse phase separations relevant to the biochemist, and accommodates both photodiode array and tunable UV/visible detection.



Waters 625-line LC system.

Waters says the system has a 0.01 to 5 ml min⁻¹ flow range capability and a 4,000 p.s.i. operating limit. Special features include an automated plunger wash, four-buffer blending, flexible non-metallic tubing and injector, and finger-tightened fittings. The \$19,000–29,500 (US) model 625 also provides Waters' PowerLine single point control of all gradient, injection, detection and external event parameters, with or without a computer.

Eldex has a new integrated, programmable **solvent delivery system** for applications ranging from microbore HPLC separations to high-resolution preparative-scale purifications (*Reader Service*



Programmable solvent delivery from Eldex. No. 109). Eldex's model 9600 solvent delivery system features interactive menus for methods development without the need for programming skills. The unit will generate ternary gradients with pulse-free solvent delivery, and has input and output ports for interfacing with other chromatography peripherals. Eldex sells the instrument for \$6,995 (US).

Nucleotide news

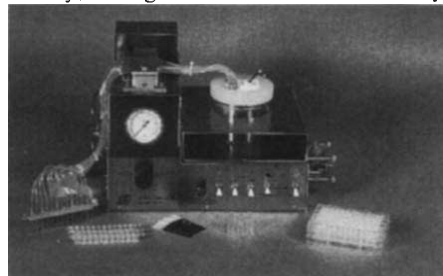
British Bio-technology Ltd sells a line of **DNA probes for interleukins** by the name of Designer Probes, after its successful line of Designer Genes (*Reader Service No. 110*). The Designer Probes recognize certain unique exons of genes coding for interleukins, and their mRNA. The probes are available for three or more exons within the genes for interleukin-1 α , interleukin-1 β , and interleukins 2–6. Probe cocktails consisting of an equimolar mixture of exon-specific probes are also available, to detect different regions of a gene simultaneously, yielding higher sensitivity. British Bio-technology Ltd also has a new **cytokine sampler kit** which contains trial-size samples of recombinant human cytokines interleukin-1 α , -1 β , -2, -3, -4, -6, and tumour necrosis factors α and β . The cytokines are produced from BBL's Designer Genes by R + D Systems in the United States. They are distributed in North and South America by R + D Systems, and in Japan by Funakoshi Pharmaceutical.

Version 5.0 of Hitachi's HIBIO
NATURE · VOL 338 · 16 MARCH 1989

DNASIS and PROSIS **sequence-manipulation software** has just been released (*Reader Service No. 111*). The new versions can perform homology searches three times faster than older releases, can create DXF-formatted output files, and can support HP Laserjet printers. DNASIS now contains two completely new analytical functions: protein coding region prediction based on Fickett's method; and secondary structure, nucleic acid sequence prediction based on Zuker's method. Restriction maps can now be displayed in circular and linear formats, together on the screen with the fragment, its size and map location. PROSIS now supports the parameter set for Rose and Roy hydrophilicity/hydrophobicity analyses, and can display Edmundson Wheel vectors of hydrophobic sites within a sequence. PROSIS and DNASIS cost \$1,600 (US) each; current users can upgrade their versions for \$400 (US) each.

New in Immunology

Dynatech Laboratories has a new 12-channel **dispensing system** for filling microtitre plates, slides, cards, strips and vials — the Quick Spense IIIe (*Reader Service No. 112*). Built by Sandy Spring Instruments, the Quick Spense IIIe is an automated liquid delivery system which couples time pressure dispensing with precision, cam-activated valving. Up to three reagents can be dispensed simultaneously, using 1–12 channels. Delivery



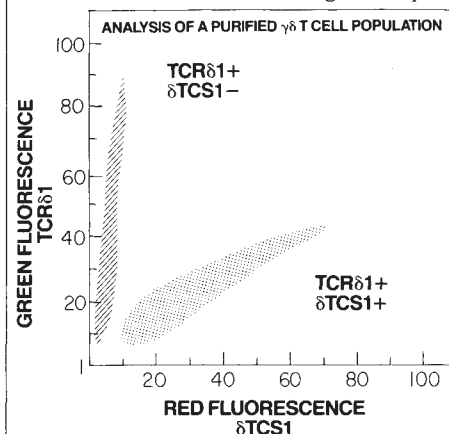
Quick Spense IIIe for multichannel delivery.

volumes range from 5 μ l to over 1 ml, and can be extended using an optional module. The dispensing head may be custom designed for any multiple dispensing format, and manual dispensing can be controlled by the use of the included foot switch or by the activation switch mounted on the control panel. Product contact parts can be flushed in place, or removed and autoclaved. Dynatech says the system has an accuracy rate of approximately 1.5 per cent for materials of standard viscosity.

Human monoclonal antibody against HIV is now available from the Swedish company BioInvent (*Reader Service No. 113*). The company develops the antibody through its patented *in vitro* immunization technology, which involves fusing peripheral lymphocytes isolated from a healthy blood donor which have been exposed to a

recombinant immunodominant peptide from gp 41 to an EBV-infected heteromyeloma. The resulting monoclonal is an IgM-kappa isotope antibody which BioInvent says has no cross-reactivity with unrelated proteins. BioInvent also offers human monoclonal antibodies specific for digoxin and cytomegalovirus.

T Cell Sciences has announced its second monoclonal antibody to the delta chain of the human T cell antigen receptor



Data from T cell Sciences.

— **Identi-T TCR δ 1** (*Reader Service No. 114*). T Cell Sciences says the **T cell antigen receptor MAb** identifies a framework

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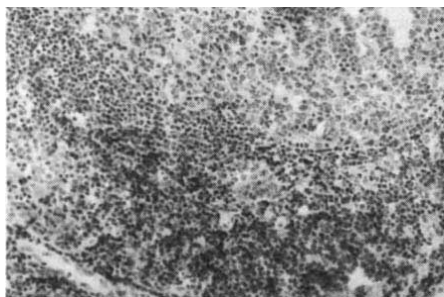
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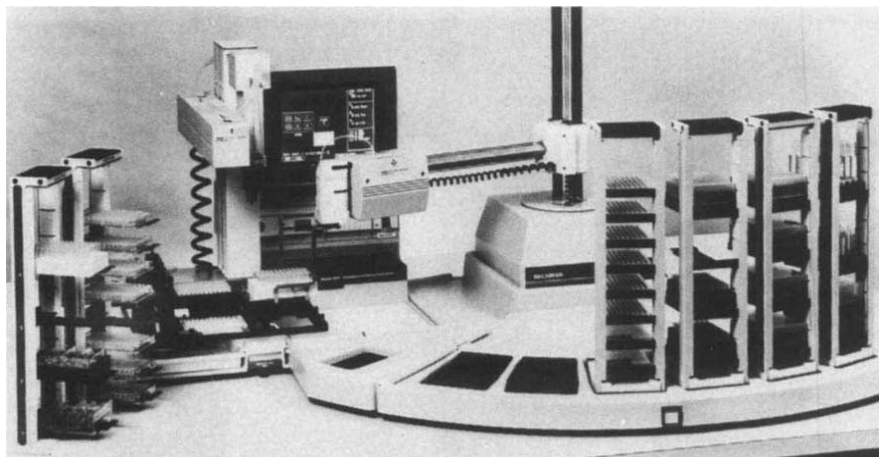
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or common determinant of the delta chain as expressed on the surface of gamma-delta positive peripheral blood lymphocytes, interleukin-2 cultured thymocytes, specific T cell lines and in tissue sections. Identi-T TCR61 is available in purified, FITC conjugated and biotinylated forms for use in immunofluorescent staining of live cells, immunochemical staining of frozen tissue sections and fixed cells, and immunoprecipitation. T Cell Sciences also sells a monoclonal specific for the alpha chain of the T cell antigen receptor. Indenti-T α F1 binds to a nonpolymorphic determinant of the alpha chain, normally hidden on viable cells but exposed on fixed cells and frozen tissue sections. It also binds to T cell antigen receptor alpha-beta heterodimers. Identi-T α F1 offers an alternative to mRNA detection for study-



The Biomek 1000 automated workstation with its robotic arm.

ing the ontogeny of T cells, and can be used in Western blot analyses.

Beckman Instruments' Biomek 1000 automated laboratory workstation can be configured for receptor binding studies (Reader Service No. 115). The \$26,500 (US) Biomek SL robotic arm can be used for loading and unloading labware onto the \$24,000 (US) Biomek 1000 system. The system includes wedge-shaped stack areas which hold up to six stacks of labware — such as pipette tips, multiwell plates and tubes — within reach of the robotic arm. The workstation automatically performs pipetting, plate washing and photometry readings. For receptor binding studies, Beckman also offers 2-ml Cube 2ubes in a 96-well format, and deep-well 96-well microtitre plates with a single well capacity of 1 ml. Both Cube 2ubes and deep-well microtitre plates come sterile or unsterile, with or without covers.

GIBCO Laboratories has a new **protein-free hybridoma medium** to be used as an alternative to serum-supplemented media for monoclonal antibody production (Reader Service No. 116). GIBCO says its PFHM-II medium, available as powder or liquid, contains no polypeptide growth factors, attachment factors, or mediators to complicate downstream purification, and that it performs as well as serum-supplemented media in conventional and high-density culture systems.

The literature shelf

Schleicher & Schuell's new four-colour **life sciences catalogue** describes apparatus and filter media available from the company for a variety of separation techniques, transfer methods, solid phase assays and related procedures (Reader Service No. 117). Among the new products featured are Schleicher & Schuell's Profile mini-electrophoresis system, Elutrap electroseparation unit, and dual-filter Uniflo Plus syringe filter unit. A section devoted to transfer media describes new Optibind reinforced nitrocellulose membranes, and a new section outlines the company's services for developers of

test kits and device manufacturers. The free catalogue also includes a selection guide to help users choose the appropriate product for a specific application.

The new 1989 catalogue on **cell culture reagents** from Boehringer Mannheim Biochemicals features the company's Nutridoma media supplements, cell culture factors and cytokines, antibodies to factors and cytokines, dissociating and harvesting enzymes, hybridoma reagents, antibiotics and complementary products (Reader Service No. 118). The free catalogue outlines the form, purity, concentration, price and other pertinent information on the full range of Boehringer Mannheim's products for cell culture.

Stratagene's new **1989 catalogue** should hit the streets this month (Reader Service No. 119). The company's free catalogue will outline its Stratalinker for crosslinking nucleotides to membranes, its ThermalBase kit for DNA sequencing using *Taq* polymerase, and its Zap cDNA synthesis kit for uni-directional cloning. Also described will be Stratagene's custom library preparation services, its large selection of restriction and modifying enzymes, and its Epicurean Coli high efficiency competent *Escherichia coli*.

Setting it straight

The instrument for using caged neurotransmitters described in the 9 February issue (*Nature* 337, 583; 1989) was developed while the author was at the School of Applied and Engineering Physics, Cornell University. Jeffrey Marque is now with the Spinco Division of Beckman Instruments. The article also should have referred to work on the measurement of light-activated neurotransmitters by H. A. Lester and J. M. Norbonne (*A. Rev. biophys. Bioengng* 11, 151-175, 1982). □

These notes are compiled by Carol Ezzell from information compiled by the manufacturers. To obtain further details, fill out the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.

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